Europäisches Patentamt

European Patent Office

Office européen des brevets

Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

02077953.4

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Anmeldung Nr:
Application no.: 02077953.4
Demande no:

Anmeldetag:
Date of filing: 18.07.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Crucell Holland B.V.
Archimedesweg 4
2333 CN Leiden
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Recombinant production of mixtures of antibodies

In Anspruch genommene Prioriät(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N15/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SK TR

EPO - DG 1

18. 07. 2002

Title: Recombinant production of mixtures of antibodies

(55)

Field of the invention

The present invention relates to the field of medicine, more in particular to the field of production of antibodies, more in particular to the production of mixtures of antibodies.

Background of the invention

The essential function of the immune system is the defence against infection. The humoral immune system combats molecules recognized as non-self, such as pathogens, using immunoglobulines. These immunoglobulines, also called antibodies, are raised specifically against the infectious agent, which acts as an antigen, upon first contact (Roitt, Essential Immunology, Blackwell Scientific Publications, fifth edition, 1984; all references cited herein are incorporated in their entirety by reference). Antibodies are multivalent molecules comprising heavy (H) chains and light (L) chains joined with interchain disulfide bonds. Several isotypes of antibodies are known, including IgG1, IgG2, IgG3, IgG4, IgA, IgD, IgE, and IgM. An IgG contains two heavy and two light chains. Each chain contains constant (C) and variable (V) regions, which can be broken down into domains designated C_{H1}, C_{H2}, C_{H3}, V_H, and C_L, V_L (Fig. 1). Antibody binds to antigen via the variable region domains contained in the Fab portion, and after binding can interact with molecules and cells of the immune system through the constant domains, mostly through the Fc portion.

B-lymphocytes can produce antibodies in response to exposure to biological substances like bacteria, viruses and their toxic products. Antibodies are generally epitope specific and bind strongly to biological substances carrying these

epitopes. The hybridoma technique (Kohler and Milstein 1975) makes use of the ability of B-cells to produce monoclonal antibodies to specific antigens and to subsequently produce these monoclonal antibodies by fusing B-cells from mice exposed to the antigen of interest to immortalized murine plasma cells. This technology resulted in the realization that monoclonal antibodies produced by hybridoma's could be used in research, diagnostics and therapies to treat different kinds of diseases like cancer and auto-immune related disorders.

Because antibodies that are produced in mouse hybridoma's induce strong immune responses in humans, it has been appreciated in the art that antibodies required for successful treatment of humans needed to be less or preferably non-immunogenic. For this, murine antibodies were first engineered by replacing the murine constant regions with human constant regions (referred to as chimeric antibodies). Subsequently, domains between the complementarity determining regions (CDRs) in the variable domains, the so-called framework regions, were replaced by their human counterparts (referred to as humanized antibodies). The final stage in this humanization process has been the production of fully human antibodies.

In the art, also bispecific antibodies, which have binding specificities for two different antigens, have been described. These are generally used to target a therapeutic or diagnostic moiety, e.g. T-cell, a cytotoxic trigger molecule or a chelator that binds a radionuclide, that is recognized by one variable region of the antibody to a cell that is recognized by the other variable region of the antibody, e.g. a tumor cell (see for bispecific antibodies Le Doussal et al, 1992; Kroesen et al, 1993, 1994; Weiner, 2000,

Fanger et al, 1991; Segal et al, 1999, 2001; Weiner and De Gast, 1995).

One very useful method known in the art to obtain fully human monoclonal antibodies with desirable binding properties employs phage display libraries. This is an *in vitro*, recombinant DNA-based, approach that mimics key features of the humoral immune response (see for phage display methods e.g. McCafferty et al, 1990; Marks et al, 1991, 1993; Hoogenboom and Winter, 1992; Chester et al, 1994; Burton and Barbas 1994; Winter et al, 1994; De Kruif et al, 1995a, 1995b; Harrison et al, 1996; WO 90/02809; WO 92/01047; WO 93/19172; WO 93/11236; WO 93/06213; US patent 6,265,150). For the construction of phage display libraries, collections of human monoclonal antibody heavy and light chain variable region genes are expressed on the surface of bacteriophage particles, usually in single chain Fv (scFv) or in Fab format. Large libraries of antibody fragment-expressing phages typically contain more than 10^9 antibody specificities and may be assembled from the immunoglobulin V regions expressed in the B lymphocytes of immunized- or non-immunized individuals. Alternatively, phage display libraries may be constructed from immunoglobulin variable regions that have been partially assembled or rearranged *in vitro* to introduce additional antibody diversity in the library (semi-synthetic libraries) (Hoogenboom and Winter, 1992; De Kruif et al, 1995b). For example, *in vitro* assembled variable regions contain stretches of synthetically produced, randomized or partially randomized DNA in those regions of the molecules that are important for antibody specificity. The genetic information encoding the antibodies identified by phage display, can be used for cloning the antibodies in a desired

format, e.g. IgG, IgA or IgM, to produce the antibody with recombinant DNA methods (Boel et al, 2000).
An alternative method to provide fully human antibodies uses transgenic mice that comprise genetic material encoding a human immunoglobulin repertoire (Fishwild et al, 1996; Mendez et al, 1997). Such mice can be immunized with a target antigen, and the resulting immune response will produce fully human antibodies. The sequences of these antibodies can be used in recombinant production methods.

Production of monoclonal antibodies routinely is performed by use of recombinant expression of the nucleic acid sequences encoding the H and L chains of antibodies in host cells (see e.g. EP0120694; EP0314161; EP0481790; US patent 4,816,567; WO 00/63403).

To date, many different diseases are being treated with either humanized or fully human monoclonal antibodies. Products based on monoclonal antibodies that are currently approved for use in humans include Herceptin™ (anti-Her2/Neu), Reopro™ (anti-Glycoprotein IIB/IIIA receptor), Mylotarg™ (anti-CD33), Rituxan™ (Rituximab, anti-CD20), Simulect™ (anti-CD25), Remicade™ (anti-TNF), Synagis™ (anti-RSV), Zenapax™ (IL2-receptor), CAMPATH™ (anti-CD52). Despite these successes, there is still room for new antibody products and for considerable improvement of existing antibody products. The use of monoclonal antibodies in cancer treatment has shown that so-called "antigen-loss tumor variants" can arise, making the treatment with the monoclonal antibody less effective. Treatment with the very successful monoclonal antibody Rituximab (anti-CD20) has for instance shown that antigen-loss escape variants can occur, leading to relapse of the lymphoma (Kinoshita et al, 1998; Schmitz et al, 1999; Massengale et al, 2002). In the art, the potency of

monoclonal antibodies has been increased by fusing them to toxic compounds, such as radionuclides, toxins, cytokines, and the like. Each of these approaches however has its limitations, including technological and production problems and/or high toxicity.

Furthermore, it appears that the gain in specificity of monoclonal antibodies compared to traditional undefined polyclonal antibodies, goes at the cost of loss of efficacy. In vivo, antibody responses are polyclonal in nature, i.e. a mixture of antibodies is produced because various B-cells respond to the antigen, resulting in various specificities being present in the polyclonal antibody mixture. Polyclonal antibodies can also be used for therapeutic applications, e.g. for passive vaccination or for active immunotherapy, and currently are usually derived from pooled serum from immunized animals or from humans who recovered from the disease. The pooled serum is purified into the proteinaceous or gamma globulin fraction, so named because it contains predominantly IgG molecules. Polyclonal antibodies that are currently used for treatment include anti-rhesus polyclonal antibodies, gamma-globulin for passive immunization, anti-snake venom polyclonal (CroFab), Thymoglobulin™ for allograft rejection, anti-digoxin to neutralize the heart drug digoxin, and anti-rabies polyclonal. In currently marketed therapeutic antibodies, an example of the higher efficacy of polyclonal antibodies compared to monoclonal antibodies can be found in the treatment of acute transplant rejection with anti-T-cell antibodies. The monoclonal antibodies on the market (anti-CD25 basiliximab) are less efficacious than a rabbit polyclonal antibody against thymocytes (Thymoglobulin™) (press releases dated March 12 and April 29, 2002, on www.sangstat.com). The use of pooled

human sera however potentially bears the risk of infections with viruses such as HIV or hepatitis, with toxins such as lipopolysaccharide, with proteinaceous infectious agents such as prions, and with unknown infectious agents. Furthermore, the supply that is available is limited, and insufficient for widespread human treatments. Problems associated with the current application of polyclonal antibodies derived from animal sera in the clinic include a strong immune response of the human immune system against such foreign antibodies. Therefore such polyclonals are not suitable for repeated treatment, or for treatment of individuals that were injected previously with other serum preparations from the same animal species.

The art describes the idea of the generation of animals with a human immunoglobulin repertoire, which can subsequently be used for immunization with an antigen to obtain polyclonal antibodies against this antigen from the transgenic animals (e.g. see WO 00/46251; WO 01/19394; Ishida et al, 2002). However, much technological hurdles still will have to be overcome before such a system is a practical reality in larger animals than mice, and it will take years of development before such systems can provide the polyclonal antibodies in a safe and consistent manner in sufficient quantities. Moreover, antibodies produced from pooled sera, whether being from human or animal origin, will always comprise a high amount of unrelated and undesired specificities, as only a small percentage of the antibodies present in a given serum will be directed against the antigen used for immunization. It is for instance known that in normal, i.e. non-transgenic, animals about 1-10% of the circulating immunoglobulin fraction is directed against the

antigen used for hyper-immunization, hence the vast majority of circulating immunoglobulins is not specific.

One approach towards expression of polyclonal antibody libraries has been described (Den et al, 1999; Santora et al, 2000; Baecher-Allan et al, 1999; WO 95/20401; US patents 5,789,208 and 6,335,163). A polyconal library of Fab antibody fragments is expressed using a phage display vector, and selected for reactivity towards an antigen. The selected heavy and light chain variable region gene combinations are transferred in mass, as linked pairs, to a eukaryotic expression vector that provides constant region genes, to obtain a sublibrary of intact polyconal antibodies. Upon transfection of this sublibrary into myeloma cells, stable clones produce monoclonal antibodies that can be mixed to obtain a polyclonal antibody mixture. While in theory it would be possible to obtain polyclonal antibodies directly from a single recombinant production process using this method by culturing a mixed population of transfected cells, potential problems would occur concerning the stability of the mixed cell population, and hence the consistency of the produced polyclonal antibody mixture. The control of a whole population of different cells in a pharmaceutically acceptable large (*i.e.* industrial) scale process, is a daunting task. It would seem, that characteristics such as growth rates of the cells and production rates of the antibodies should remain stable for all the individual clones of the non-clonal population in order to keep the ratio of antibodies in the polyclonal antibody mixture more or less constant. Thus, while the need for mixtures of antibodies may have been recognized in the art, no acceptable solutions exist to make mixtures of antibodies economically in a pharmaceutically acceptable way. It is the object of the

present invention to provide novel means for producing a mixture of antibodies in recombinant hosts.

Brief description of the figures

Fig. 1 Schematic representation of an antibody. The heavy and light chains are paired via interchain disulfide bonds (dotted lines). The heavy chain can be either of the $\alpha, \gamma, \omega, \delta$ or ε isotype. The light chain is either λ or κ. An antibody of IgG1 isotype is shown.

Fig. 2 Schematic representation of a bi-specific monoclonal antibody. A bi-specific antibody contains two different fuctional F(Ab) domains, indicated by the different patterns of the V_H-V_L regions.

Fig. 3 Sequence alignment of V_L and V_H of K53 and UBS-54.

Fig. 4 Overview of plasmids pUBS3000Neo and pCD46_3000 (Neo).

Fig. 5 A. IEF of transiently expressed pUBS3000Neo, pCD46_3000(Neo) and a combination of both. B. The upper part shows a schematic representation of the expected molecules when a single light chain and a single heavy chain are expressed in a cell, leading to monoclonal antibodies UBS-54 or K53. The lower part under the arrow shows a schematic representation of the combinations produced when both heavy chains and the common light chain are co-expressed in a host cell, with theoretical amounts when both heavy chains are expressed at equal levels and pair to each other with equal efficiency. The common light chain is indicated with the vertically striped bars.

Fig. 6. Schematic representation of the method according to the invention (see e.g. Example 2). Nucleic acid sequences encoding one light chain and three different heavy chains capable of pairing to the common light chain to give functional antibodies are introduced into host cells, and after selection of stable clones, the clones can be screened for e.g. expression levels, binding, production of functional mixtures of antibodies.

Fig. 7. Sequence of V_H and V_L of phages directed against CD22, CD72 and HLA-DR.

Fig. 8. Map and DNA sequence of pCRU-L01.

Fig. 9. Map of pUBS54-IgA (pCRU-L01 encoding human IgA1 against EPCAM).

Fig. 10. Dimeric bi-specific IgA with a single light chain. The method of the invention will produce a mixture of forms wherein different heavy chains can be paired, and only the most simple form is depicted in this schematic representation. The J-chain is shown to join the two monomers.

Fig. 11. Pentameric multispecific IgM with a single light chain. The method of the invention will produce a mixture of many different forms wherein different heavy chains can be paired, and only the most simple form when 5 different heavy chains are expressed with a single light chain, and all 5 different heavy chains are incorporated in the pentamer and paired to the same heavy chain, is depicted in this schematic representation. Pentamers with less specificities can also be

formed by incorporation of less than 5 different heavy chains. When the J-chain is not expressed, hexamers can also be obtained.

Fig. 12. Expression of a mixture of human IgG isotypes consisting of a common light chain, but with different binding specificities in a single cell to avoid the formation of bispecific antibodies. The different binding specificities are indicated by the different colours of the VH sequences. The common light chain is indicated with the vertically striped bars. The IgG1 isotype is indicated with the non-striped Fc, the IgG3 isotype is indicated with the horizontally striped Fc part.

Fig. 13. Map and DNA sequence of pCRU-K03.

Summary of the invention

In one aspect the present invention provides for a method of producing a mixture of antibodies in a recombinant host, the method comprising the step of:

expressing in a recombinant host cell a nucleic acid sequence or nucleic acid sequences encoding at least one light chain and at least three different heavy chains that are capable of pairing with said at least one light chain. A further aspect of the invention is the elimination of the production of potentially non-functional light-heavy chain pairing by using pre-selected combinations of heavy and light chains. It has been recognized that phage display libraries built from a single light chain and many different heavy chains can encode antibody fragments with very distinct binding properties. This feature can be used to find different antibodies having the same light chain but different heavy chains, against the same target or different targets, wherein a target can be a whole antigen or an epitope thereof. Such different targets may for instance be on the same surface (e.g. cell or tissue). Such antibody fragments obtained by phage display can be cloned into vectors for the desired format, e.g. IgG, IgA or IgM, and the nucleic acid sequences encoding these formats can be used to transfect host cells. In one approach, H and L chains can be encoded by different constructs that, upon transfection into a cell wherein they are expressed, give rise to intact Ig molecules. When different H chain constructs are transfected into a cell with a single L chain construct, H- and L-chains will be assembled to form all possible combinations. However, in contrast to approaches where different light chains are expressed such as for the production of bispecific antibodies, this method will result only in functional binding regions. It would be particularly

useful when the host, *e.g.* a single cell line, is capable of expressing acceptable levels of recombinant antibodies, without the necessity to first amplify in said cell the nucleic acid sequences encoding the antibodies. The advantage is that cell lines with only a limited copy number of said nucleic acids are expected to be genetically more stable, because there will be less recombination between the sequences encoding the heavy chains, than in cell lines where a multitude of these copies is present. A cell line suitable for use in the methods according to the invention is the human cell line PER.C6™. Using this method a mixture of antibodies with defined specificities can be produced from a single cell clone in a safe, controlled, and consistent manner.

The invention provides a method for production of a mixture of antibodies in a recombinant host, the method comprising the step of: expressing a nucleic acid sequence or nucleic acid sequences encoding at least one light chain and at least three different heavy chains that are capable of pairing with said at least one light chain in a recombinant host cell. In a preferred aspect, the recombinant host cell comprises a nucleic acid sequence encoding a common light chain that is capable of pairing with said at least three different heavy chains, such that the produced antibodies comprise a common light chain. Obviously, those of skill in the art will recognize that "common" also refers to functional equivalents of the light chain of which the amino acid sequence is not identical. Many variants of said light chain exist wherein mutations (deletions, substitutions, additions) are present that do not materially influence the formation of functional binding regions.

The invention further provides a composition comprising a mixture of recombinantly produced antibodies, wherein at least three different heavy chain sequences are represented in said mixture. In one embodiment, the light chains of such mixtures have a common sequence. The mixture of antibodies can be produced by the method according to the invention. Preferably, the mixture of antibodies is more efficacious than the individual antibodies it comprises, more preferably, the mixture acts synergistically in a functional assay.

The invention further provides a recombinant host cell for producing mixtures of antibodies, and methods for making such host cells.

Independent clones obtained from the transfection of nucleic acid sequences encoding a light chain and more than one heavy chain may express the different antibodies in the mixture at different levels. It is another aspect of the invention to select a clone using a functional assay, for the most potent mixture of antibodies. The invention therefore further provides a method for identifying at least one host cell clone that produces a mixture of antibodies, wherein said mixture of antibodies has a desired effect according to a functional assay, the method comprising the steps of: (i) providing a host cell with nucleic acid sequence encoding at least one light chain and nucleic acid sequences encoding at least two different heavy chains, wherein said heavy and light chains are capable of pairing with each other; (ii) culturing at least one clone of said host cell under conditions conducive to expression of said nucleic acid sequences; (iii) screening said at least one clone of the host cell for production of a mixture of antibodies having the desired effect by a functional assay; and (iv) identifying at least one clone that produces a mixture of

antibodies having the desired effect. This method according to the invention can be performed using high-throughput procedures, if desired. The clones identified by the method can be used to produce antibody mixtures according to the invention.

The invention further provides transgenic non-human animals and transgenic plants or transgenic plant cells capable of expressing mixtures of antibodies, and mixtures of antibodies produced by these.

The invention further provides pharmaceutical compositions comprising a mixture of recombinantly produced antibodies and a suitable carrier.

The invention further provides mixtures of antibodies for use in the treatment or diagnosis and for the preparation of a medicament for use in the treatment or diagnosis of a disease or disorder in a human or animal subject.

The invention further provides a method for producing a mixture of antibodies comprising different isotypes from a single host cell clone.

The invention further provides a method for indentifying a mixture of antibodies having a desired effect in a functional assay.

The invention further provides a method for producing a mixture of antibodies that are capable of binding to a target, the method comprising the steps of: i) bringing a phage library comprising antibodies into contact with material comprising a target, ii) at least one step of selecting phages binding to said target, iii) identifying at least two phages that comprise antibodies binding to said target, wherein said at least two antibodies comprise a common light chain, iv) introducing a nucleic acid sequence encoding the light chain and a nucleic acid sequence or

sequences encoding the heavy chains of said at least two antibodies into a host cell,

v) culturing a clone of said host cell under conditions conducive to expression of said nucleic acid sequences.

Detailed description of the invention

It is an object of the present invention to provide a method for producing a mixture of antibodies in a recombinant host, the method comprising the step of: expressing in a recombinant host cell a nucleic acid sequence or nucleic acid sequences encoding at least one light chain and at least three different heavy chains that are capable of pairing with said at least one light chain. According to the invention, the light and heavy chains when paired form functional antigen binding domains. A functional antigen binding domain is capable of specifically binding to an antigen.
In one aspect according to the invention, the method for producing a mixture of antibodies according to the invention further comprises the step of recovering the antibodies from the cell or the host cell culture to obtain a mixture of antibodies suitable for further use. In one embodiment of the invention, a method is provided for production of a mixture of antibodies, the method comprising the step of: expressing in a recombinant host cell a nucleic acid sequence encoding a common light chain and nucleic acid sequence or sequences encoding at least three different heavy chains that are capable of pairing with said common light chain, such that the antibodies that are produced comprise common light chains. In one aspect the common light chain is identical in each light chain/heavy chain pair.

The term "antibody" as used herein means a polypeptide containing one or more domains that bind an epitope on an antigen, where such domains are derived from or have sequence identity with the variable region of an antibody. The structure of an antibody is schematically represented in Fig.1. Examples of antibodies according to the invention include full length antibodies, antibody fragments,

bispecific antibodies, immunoconjugates, and the like. An antibody according to the invention may be isotype IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, IgM, and the like, or a derivative of these. Antibody fragments include Fv, Fab, Fab', $F(ab')_2$ fragments, and the like. Antibodies according to the invention can be of any origin, including murine, of more than one origin, *i.e.* chimeric, humanized, or fully human antibodies. Immunoconjugates comprise antigen binding domains and a non-antibody part such as a toxin, a radiolabel, an enzyme, and the like. An "antigen binding domain" preferably comprises variable regions of a heavy and a light chain, and is responsible for specific binding to an antigen of interest. Recombinant antibodies are prepared by expressing both a heavy and a light chain in a host cell. Similarly, by expressing two chains with their respective light chains (or a common light chain), wherein each heavy chain/light chain has its own specificity, so-called "bispecific" antibodies can be prepared. "Bispecific antibodies" comprise two non-identical heavy-light chain combinations (Fig. 2), and both antigen binding regions of a bispecific antibody may recognize different antigens or different epitopes on an antigen.

A "common light chain" according to the invention refers to light chains which may be identical or have amino acid sequence differences. Said light chains may comprise mutations which do not alter the specificity of the antibody when combined with the same heavy chain, without departing from the scope of the present invention. It is for instance possible within the scope of the definition of common light chains as used herein, to prepare or find light chains that are not identical but still functionally equivalent, e.g. by introducing and testing conservative amino acid changes,

changes of amino acids in regions that do not or only partly contribute to binding specificity when paired with the heavy chain, and the like. It is an aspect of the present invention to use as common light chain one identical light chain to combine with different heavy chains to form antibodies with functional antigen binding domains. The use of one identical light chain avoids the formation of heterodimers in which pairing of light and heavy chains results in antigen binding domains that are not functional, in other words which are not capable of binding to the target antigen or antigens. The use of a common light chain and two heavy chains has been proposed by (Merchant et al, 1998; WO 98/50431) for a different purpose, viz. to *increase* the formation of functional bispecific antibodiesat the expense of antibody mixture complexity. These publications teach a method for preferentially producing one defined and desired bispecific antibody, thereby *minimizing* the complexity of the produced mixture. Hence, Merchant specifically teaches to prevent the production of monospecific antibodies, because these are undesired byproducts in the process for bispecific antibody production described in those publications. Clearly, there is no teaching in the prior art to prepare a complex mixture of antibodies from a recombinant host cell avoiding the formation of non-functional binding domains or the benefits thereof, let alone how. In the method according to the present invention, at least three different heavy chains that are capable of pairing with the common light chain are expressed. In other embodiments, the host cell according to the invention is provided with nucleic acid sequences encoding for 4, 5, 6, 7, 8, 9, 10, or more heavy chains capable of pairing with the common light chain, to increase the complexity of the produced mixture of antibodies.

"Different heavy chains" according to the invention may differ in the variable region and have the same constant region. In other embodiments, where it is clear from the context, they may have the same variable region and differ in the constant region, e.g. be of a different isotype. The use of a mixture of antibodies having different constant regions, such as the Fc-portion, may be advantageous if different arms of the immune system are to be mobilized in the treatment of the human or animal body. In yet other embodiments, also to be clear from the context, both the variable and the constant regions may differ.

A "mixture of antibodies" according to the invention comprises at least two non-identical antibodies, but may comprise 3, 4, 5, 6, 7, 8, 9, 10, or more different antibodies, and may resemble a polyclonal or at least an oligoclonal antibody mixture with regard to complexity and number of functional antigen binding molecules. The mixtures produced according to the present invention usually will comprise bispecific antibodies. If desired, formation of monospecific antibodies in the mixture can be favoured over the formation of bispecific antibodies. When n heavy chains and one common light chain are expressed according to the invention in a host cell at equal levels, the theoretical percentage of bispecific antibodies produced by the method according to the invention is $(1-1/n)*100\%$. The total number of different antibodies in the mixture produced by the method according to the invention is theoretically $n+\{(n^2-n)/2\}$, of which $(n^2-n/2)$ are bispecific antibodies. Distortion of the ratio of expression levels of the different heavy chains may lead to values deviating from the theoretical values. The amount of bispecific antibodies can also be decreased, compared to these theoretical values, if not all heavy chains

pair with equal efficiency. It is for instance possible to engineer the heavy chains, *e.g.* by introducing specific and complementary interaction surfaces between selected heavy chains, to promote homodimer pairing over heterodimer pairing, contrary to what has been proposed by Merchant, *supra*. Heavy chains may also be selected so as to minimize heterodimer formation in the mixture. A special form of this embodiment involves heavy chains of two or more different isotypes (e.g. IgG1, IgG3, IgA). When heavy chains of different isotype are expressed in the same host cell in accordance with the present invention and one light chain that can pair to these heavy chains, the amount of bispecific antibodies will be reduced, possibly to very low or even to undetectable levels. Thus, when bispecific antibodies are less desirable, it is possible to produce a mixture of antibodies according to the invention, wherein a nucleic acid sequence encoding a common light chain and nucleic acid sequences encoding at least two different heavy chains with a different variable region capable of pairing to said common light chain are expressed in a recombinant host, and wherein said heavy chains further differ in their constant regions sufficiently to reduce or prevent pairing between the different heavy chains. The mixtures of antibodies according to the invention may be produced from a clone that was derived from a single host cell, i.e. from a population of cells containing the same recombinant nucleic acid sequences.

It will be understood that the different heavy chains according to the invention can be encoded on separate nucleic acid molecules, but may also be present on one nucleic acid molecule comprising different regions encoding said at least three heavy chains. The nucleic acid molecules usually encode precursors of the light and/or heavy chains, which when

expressed are secreted from the host cells, thereby becoming processed to yield the mature form. These and other aspects of expressing antibodies in a host cell are well known to those having ordinary skill in the art.

A "recombinant host cell" as used herein is a cell comprising one or more so-called transgenes, i.e. recombinant nucleic acid sequences not naturally present in said cell. These transgenes are expressed in said host cell to produce recombinant antibodies encoded by these nucleic acid sequences, when these cells are cultured under conditions conducive to expression of said nucleic acid sequences. The host cell according to the invention can be present in the form of a culture from a clone that is derived from a single host cell wherein the transgenes have been introduced. To obtain expression of nucleic acid sequences encoding antibodies, it is well known to those skilled in the art that sequences capable of driving such expression can be functionally linked to the nucleic acid sequences encoding the antibodies. Functionally linked is meant to describe that the nucleic acid sequences encoding the antibody fragments or precursors thereof is linked to the sequences capable of driving expression such that these sequences can drive expression of the antibodies or precursors thereof. Useful expression vectors are available in the art, e.g. the pcDNA vector series of Invitrogen. Where the sequence encoding the polypeptide of interest is properly inserted with reference to sequences governing the transcription and translation of the encoded polypeptide, the resulting expression cassette is useful to produce the polypeptide of interest, referred to as expression. Sequences driving expression may include promoters, enhancers and the like, and combinations thereof. These should be capable of functioning in the host cell,

thereby driving expression of the nucleic acid sequences that are functionally linked to them. Promoters can be constitutive or regulated, and can be obtained from various sources, including viruses, prokaryotic, or eukaryotic sources, or artificially designed. Expression of nucleic acids of interest may be from the natural promoter or derivative thereof or from an entirely heterologous promoter. Some well-known and much used promoters for expression in eukaryotic cells comprise promoters derived from viruses, such as adenovirus, e.g. the E1A promoter, promoters derived from cytomegalovirus (CMV), such as the CMV immediate early (IE) promoter, promoters derived from Simian Virus 40 (SV40), and the like. Suitable promoters can also be derived from eucaryotic cells, such as methallothionein (MT) promoters, elongation factor 1α (EF-1α) promoter, actin promoter, an immunoglobulin promoter, heat shock promoters, and the like. Any promoter or enhancer/promoter capable of driving expression of the sequence of interest in the host cell is suitable in the invention. In one embodiment the sequence capable of driving expression comprises a region from a CMV promoter, preferably the region comprising nucleotides -735 to +95 of the CMV immediate early gene enhancer/promoter. Protein production in recombinant host cells has been extensively described, e.g. in Current Protocols in Protein Science, 1995, Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT, ISBN 0-471-11184-8; Bendig, 1988. Culturing a cell is done to enable it to metabolize, and/or grow and/or divide and/or produce recombinant proteins of interest. This can be accomplished by methods well known to persons skilled in the art, and includes but is not limited to providing nutrients for the cell. The methods comprise growth adhering to surfaces, growth in suspension, or combinations thereof.

Several culturing conditions can be optimized by methods well known in the art to optimize protein production yields. Culturing can be done for instance in dishes, roller bottles or in bioreactors, using batch, fed-batch, continuous systems, hollow fiber, and the like. In order to achieve large scale (continuous) production of recombinant proteins through cell culture it is preferred in the art to have cells capable of growing in suspension, and it is preferred to have cells capable of being cultured in the absence of animal- or human-derived serum or animal- or human-derived serum components. Thus purification is easier and safety is enhanced due to the absence of additional animal or human proteins derived from the culture medium, while the system is also very reliable as synthetic media are the best in reproducibility.

"Host cells" according to the invention may be any host cell capable of expressing recombinant DNA molecules, including bacteria such as Eschericia (e.g. E.coli), Enterobocter, Salmonalla, Bacillus, Pseudomonas, Streptomyces, yeasts such as S.cerevisiae, K.lactis, P.pastoris, Candida, or yarrowia, filamentous fungi such as Neurospora, Aspergillus oryzae, Aspergillus nidulans and Aspergillus niger, insect cells such as Spodoptera frugiperda SF-9 or SF-21 cells, mammalian cells such as Chinese hamster ovary (CHO) cells, BHK cells, mouse cells including SP2/0 cells and NS-0 myeloma cells, primate cells such as COS and Vero cells, MDCK cells, BRL 3A cells, hybridomas, tumor-cells, immortalized primary cells, human cells such as W138, HepG2, HeLa, HEK293, HT1080 or embryonic retina cells such as PER.C6™, and the like. Often, the expression system of choice will involve a mammalian cell expression vector and host so that the antibodies are appropriately glycosylated. A human cell line, preferably

PER.C6^{TM}, can advantageously be used to obtain antibodies with a completely human glycosylation pattern. The conditions for growing or multiplying cells (see e.g. Tissue Culture, Academic Press, Kruse and Paterson, editors (1973)) and the conditions for expression of the recombinant product may differ somewhat, and optimization of the process is usually performed to increase the product yields and/or growth of the cells with respect to each other, according to methods generally known to the person skilled in the art. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach (M. Butler, ed., IRL Press, 1991). Expression of antibodies in recombinant host cells has been extensively described in the art (see e.g. EP0120694; EP0314161; EP0481790; EP0523949; US patent 4816567; WO 00/63403. The nucleic acid molecules encoding the light and heavy chains may be present as extrachromosomal copies and/or stably integrated into the chromosome of the host cell. With regard to stability of production, the latter is preferred.

The antibodies are expressed in the cells according to the invention, and may be recovered from the cells or preferably from the cell culture medium, by methods generally known to persons skilled in the art. Such methods may include precipitation, centrifugation, filtration, size-exclusion chromatography, affinity chromatography, cation- and/or anion-exchange chromatography, hydrophobic interaction chromatography, and the like. For a mixture of antibodies comprising IgG molecules, protein A or protein G affinity chromatography can be suitably used (see e.g. US patents 4,801,687 and 5,151,504).

In one embodiment, at least two antibodies from the mixture produced according to the invention comprise a heavy-light chain dimer having different specificities and/or affinities. The specificity determines which antigen or epitope thereof is bound by the antibody. The affinity is a measure for the strength of binding to a particular antigen or epitope. Specific binding is defined as binding with an affinity (Ka) of at least 5 * 10E4 liter/mole, more preferably 5 * 10E5, more preferably more than 5 * 10E6, still more preferably 5 * 10E7, or more. Typically, monoclonal antibodies may have affinities which go up to 10E10 liter per mole, or even higher. The mixture of antibodies produced according to the present invention may contain at least two antibodies that bind to different epitopes on the same antigen molecule and/or may contain at least two antibodies that bind to different antigen molecules present in one antigen comprising mixture. Such an antigen comprising mixture may be a mixture of partially or wholly purified antigens such as toxins, membrane components and proteins, viral envelope proteins, or it may be a healthy cell, a diseased cell, a mixture of cells, a tissue or mixture of tissues, a tumor, an organ, a complete human or animal subject, a fungus or yeast, a bacteria or bacterial culture, a virus or virus stock, combinations of these, and the like. Unlike monoclonal antibodies that are able to bind to a single antigen or epitope only, the mixture of antibodies according to the present invention may therefore have many of the advantages of a polyclonal antibody mixture.

In a preferred embodiment, the host cell according to the method of the invention is capable of high-level expression of human immunoglobulin, i.e. at least 1 pg/cell/day, preferably at least 10 pg/cell/day and even more

preferably at least 20 pg/cell/day or more without the need for amplification of the nucleic acid molecules encoding the heavy and light chains in said host cell. In the art, amplification of the copy number of the nucleic acid sequences encoding a protein of interest in e.g. CHO cells can be used to increase expression levels of the recombinant protein by the cells (see e.g. Bendig, 1988; Cockett et al, 1990; US patent 4,399,216). This is currently a widely used method. However, a significant time-consuming effort is required before a clone with a desired high copy number and high expression levels has been established, and moreover clones harbouring very high copy numbers (up to hundreds) of the expression cassette often are unstable (e.g. Kim et al., 1998). It is therefore a preferred embodiment of the present invention to use host cells that do not require such amplification strategies for high-level expression of the antibodies of interest. This allows fast generation of stable clones of host cells that express the mixture of antibodies according to the invention in a consistent manner. Preferably host cells according to the method of the invention are derived from human embryonic retina cells that have been immortalized or transformed with adenoviral E1 sequences. A particularly preferred host cell according to methods of the present invention is PER.C6™ as deposited under ECACC no. 96022940, or a derivative thereof. PER.C6-derived clones can be generated fast, usually contain a limited number of copies (about 1-10) of the transgene, and are capable of high-level expression of recombinant antibodies. Therefore, such clones are expected to maintain a stable copy number over many generations, which is an advantage in the production of biopharmaceuticals. PER.C6™ cells have been extensively characterized and documented, demonstrating good process of

scaling up, suspension growth and growth factor independence. Furthermore, PER.C6™ can be incorporated into a suspension in a highly reproducible manner, making it particularly suitable for large-scale production. In this regard, the PER.C6™ cell line has been characterized for bioreactor growth, where it can grow to very high densities. The use of PER.C6™ for recombinant production of antibodies has been described in detail in publication WO 00/63403 and in Jones et al., Genetic Engineering News vol. 22, no. 10, 2002).

It is another aspect of the present invention to provide a mixture of antibodies that is obtainable by a method according to the invention. Such mixtures of antibodies are expected to be more effective than the sole components it comprises, in analogy to polyclonal antibodies usually being more effective than monoclonal antibodies to the same target. Such mixtures can be prepared against a variety of target antigens or epitopes.

It is another aspect of the present invention to provide a recombinant host cell comprising a nucleic acid sequence encoding a light chain and a nucleic acid sequence or nucleic acid sequences encoding at least three different heavy chains of an antibody, wherein said light chain and heavy chains are capable of pairing, preferably to form a functional binding domain. The paired heavy and light chain form functional antigen binding regions against the target antigen or target antigens. The host cells according to the invention are useful in the method according to the invention. They can be used to produce mixtures of antibodies according to the invention.

It is another aspect of the present invention to provide a composition comprising a mixture of recombinantly produced antibodies, wherein at least three different heavy chain sequences are represented in the mixture of recombinant antibodies. Monoclonal antibodies are routinely produced by recombinant methods. The present invention discloses mixtures of antibodies useful for diagnosis or treatment in various fields. The compositions according to the invention comprise mixtures of at least three different heavy chains paired to light chains in the form of antibodies. Preferably, the light chains of the antibodies in said mixtures have a common light chain. The mixtures may comprise bispecific antibodies. The mixtures may be produced from a clone that was derived from a single host cell, i.e. from a population of cells containing the same recombinant nucleic acid sequences. The mixtures can be obtained by methods according to the invention, or be produced by host cells according to the invention. In other embodiments, the number of heavy chains represented in said mixture is 4, 5, 6, 7, 8, 9, 10, or more. The optimal mixture for a certain purpose may be determined empirically by methods known to the person skilled in the art, or by methods provided by the present invention. Such compositions according to the invention may have several of the advantages of a polyclonal antibody mixture, without the disadvantages usually inherently associated with polyclonal antibody mixtures, because of the manner in which they are produced. It is furthermore expected that the mixture of antibodies is more efficacious than separate monoclonal antibodies. Therefore the dosage, and hence the production capacity required, may be less for the mixtures of antibodies according to the invention than for monoclonal antibodies. Furthermore, the chances of a mixture of the invention losing

its activity due to target or epitope loss is reduced, when compared to a single monoclonal antibody. In particular embodiments, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more of the antibodies present in the mixture according to the invention have different specificities. Said different specificities may be directed to different epitopes on the same antigen and/or may be directed to different antigens present in one antigen comprising mixture. A composition according to the invention further may also comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, or more antibodies having different affinities for the same epitope. Antibodies with differing affinities for the same epitope may for instance be generated by methods of affinity maturation, known to the person skilled in the art. In a particularly preferred embodiment, the composition according to the invention has an effect that is greater than the effect of each individual monospecific antibody present in said composition. Said effect can be measured in a functional assay. A "functional assay" according to the present invention is an assay that can be used to determine one or more desired parameters of the antibody or the mixture of antibodies subject to the assay conditions. Suitable functional assays may be binding assays, apoptosis assays, antibody dependent cellular cytotoxicity (ADCC) assays, complement dependent cytotoxicity (CDC) assays, inhibition of cell growth or proliferation (cytostatic effect) assays, cell killing (cytotoxic effect) assays, cell signaling assays, assays for measuring inhibition of binding of pathogen to target cell, assays to measure the secretion of vascular endothelial growth factor (VEGF) or other secreted molecules, assays for bacteriostasis, bactericidal activity, neutralization of viruses, assays to measure the attraction of components of the immune system to the site where

antibodies are bound, including in situ hybridization methods, labeling methods, and the like. Clearly, also in vivo assays such as animal models, including mouse tumor models, models of autoimmune disease, virus-infected or bacteria-infected rodent or primate models, and the like, can be used for this purpose. The efficacy of a mixture of antibodies according to the invention can be compared to individual antibodies in such models by methods generally known to the person skilled in the art.

It is another aspect of the present invention to provide a method for identifying at least one host cell clone that produces a mixture of antibodies, wherein said mixture of antibodies has a desired effect according to a functional assay, the method comprising the steps of: (i) providing a host cell comprising a nucleic acid sequence encoding at least one light chain and nucleic acid sequence or sequences encoding at least two different heavy chains, wherein said heavy and light chains are capable of pairing with each other; (ii) culturing at least one clone of said host cell under conditions conducive to expression of said nucleic acid sequences; (iii) screening said at least one clone of the host cell for production of a mixture of antibodies having the desired effect by a functional assay; and (iv) identifying at least one clone that produces a mixture of antibodies having the desired effect. Preferably, said host cell comprises a nucleic acid sequence encoding a common light chain that is capable of pairing with said at least two different heavy chains, such that produced antibodies comprise common light chains, as described above. In specific embodiments said culturing in step (ii) and said screening in step (iii) of the method is performed with at least two

clones. The method may optionally include an assay for measuring the expression levels of the antibodies that are produced, which assay may be during or after step (ii) according to the method, or later in the procedure. Such assays are well known to the person skilled in the art, and include protein concentration assays, immunoglobulin specific assays such as ELISA, RIA, DELFIA, and the like. In particular embodiments of said method according to the invention, the host cell comprises nucleic acid sequence or sequences encoding at least 3, 4, 5, 6, 7, 8, 9, 10, or more heavy chains capable of pairing with said at least one light chain. Functional assays useful for the method according to the invention may be assays for apoptosis, ADCC, CDC, cell killing, inhibition of proliferation, virus neutralization, bacterial opsonization, receptor-mediated signaling, cell signaling, bactericidal activity, and the like. Useful screening assays for anti-cancer antibodies have for instance been described in US patent 6,180,357. Such assays may also be used to identify a clone according to the method of the present invention. It is for instance possible to use enzyme linked immunosorbent assays (ELISAs) for the testing of antibody binding to their target. Using such assays, it is possible to screen for antibody mixtures that most avidly bind the target antigen (or mixture of target antigens against which the mixture of antibodies is to be tested). Another possibility that can be explored is to directly screen for cytotoxicity or cytostatic effects. It is possible that upon such a different screen, other or the same clones producing mixtures of antibodies will be chosen than with the ELISA mentioned above. The screening for cell killing or cessation of growth of cancerous cells may be suitably used according to the invention. Cell death can be measured by

various endpoints, including the absence of metabolism or the denaturation of enzymes. In one possible embodiment of the present invention, the assay is conducted by focusing on cytotoxic activity toward cancerous cells as an endpoint. For this assay, a live/dead assay kit, for example the LIVE/DEAD® Viability/Cytotoxicity Assay Kit (L-3224) by Molecular Probes, can suitably be used. Other methods of assessing cell viability, such as trypan blue exclusion, ^{51}Cr release, Calcein-AM, Alamar Blue™, LDH activity, and similar methods can also be used. The assays may also include screening of the mixture of antibodies for specificity to the desired antigen comprising tissue. The antibodies according to the invention may have a limited tissue distribution. It is possible to include testing the mixtures of antibodies against a variety of cells, cell types, or tissues, to screen for mixtures of antibodies that preferably bind to cells, cell types or tissues of interest.

When monoclonal antibodies are produced by recombinant host cells, a screening step is usually performed to assess expression levels of the individual clones that were generated. The addition of more heavy chains to produce mixtures adds a level of complexity to the production of antibodies. When host cells are transfected with nucleic acid molecules encoding the light and heavy chains that will form the mixture of antibodies desired, independent clones may arise containing the same genetic information, but nevertheless differing in expression levels, thereby producing different ratios of the encoded antibodies, giving rise to different mixtures of antibodies from the same genetic repertoire. The method according to the invention is useful for identifying a clone that produces an optimal mixture for a certain purpose.

The culturing and/or screening according to steps (ii) and (iii) respectively, may be suitably performed using high-throughput procedures, optionally in an automated fashion. Clones can e.g. be cultured in 96-well or other multi-well plates, e.g. in arrayed format, and screened for production of a desired mixture. Robotics may be suitably employed for this purpose. Methods to implement high-throughput culturing and assays are generally available and known to the person skilled in the art. It will be clear that also for this method according to the invention, it is beneficial to use host cells capable of high level expression of proteins, without the need for amplification of the nucleic acid encoding said proteins in said cell. In one embodiment, said host cell is derived from a human embryonic retinoblast cell, that has been immortalized or transformed by adenoviral E1 sequences. In a preferred embodiment, said cell is derived from PER.C6™. This cell line has already been shown to be amenable to high-throughput manipulations, including culturing (WO 99/64582).

In specific embodiments of the present invention, said mixture of antibodies according to the method of identifying at least one host cell according to the invention, comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10, or more antibodies having different specificities and/or affinities.

A potential advantage of the method will be that it will allow exploring many possible combinations simultaneously, the combinations inherently including the presence of bispecific antibodies in the produced mixture. Therefore more combinations can be tested than by just mixing purified known monoclonal antibodies, both in number of combinations and in ratios of presence of different antibodies in these combinations.

The clone that has been identified by the method according to the invention, can be used for producing a desired mixture of antibodies. It is therefore another aspect of the present invention to provide a method of producing a mixture of antibodies, the method comprising the step of: culturing a host cell clone identified by the method of identifying at least one host cell clone that produces a mixture of antibodies according to the invention, said culturing being under conditions conducive to expression of the nucleic acid molecules encoding the at least one light chain and the at least two different heavy chains. The produced antibodies may be recovered from the host cells and/or from the host cell culture, e.g. from the culture medium. The mixture of antibodies can be recovered according to a variety of techniques known to the person skilled in the art.

It is yet another aspect of the present invention to provide a mixture of antibodies that is obtainable by the method according to the invention, described above. Said mixtures can be used for a variety of purposes, such as in treatment or diagnosis of disease, and may replace, or be used in addition to, monoclonal or polyclonal antibodies.

The methods according to the present invention may suitably use nucleic acid molecules for encoding the antibodies, which nucleic acid molecules have been obtained by any suitable method, including in vivo, *e.g.* immunization, methods or in vitro, *e.g.* antibody display methods (Amstutz et al, 2001; Mössner and Plückthun, 2001; A. Plückthun et al, In vitro selection and evolution of proteins. In: Adv. Prot. Chem., F.M. Richards et al, Eds, Academic Press, San Diego, 2001, vol 55: 367-403), such as phage display, ribosome

display or mRNA display (e.g. C. Schaffitzel et al., In vitro selection and evolution of protein-ligand interactions by ribosome display. In: Protein-Protein Interactions. A Molecular Cloning Manual, E. Golemis, Ed., Cold Spring Harbor Laboratory Press, New York, 2001, pp 535-567; Schaffitzel et al, 1999; WO 98/54312; WO 01/75097), and yeast display (e.g. WO 99/36569). Methods of identifying antibodies to a certain target, which target may be a known antigen or an unknown antigen present in an antigenic mixture, by phage display are known to the person skilled in the art. In general, a library of phages that express an antigen binding domain or derivative thereof on their surface, said antigen binding domain encoded by genetic material present in said phages, is incubated with the antigen or antigen mixture of interest, after which binding of a subpopulation of the phages that display antigen binding sites binding to the desired antigen is obtained whereas the non-binding phages are discarded. Such selection steps may be repeated one, two, or more times to obtain a population of phages that are more or less specific for the antigen of interest. Phage display methods to obtain antibodies, parts or derivatives thereof have been extensively described in e.g. McCafferty et al, 1990; Marks et al, 1991, 1993; Hoogenboom and Winter, 1992; Chester et al, 1994; Burton and Barbas 1994; Winter et al, 1994; De Kruif et al, 1995a, 1995b; Harrison et al, 1996; WO 90/02809; WO 92/01047; WO 93/19172; WO 93/11236; WO 93/06213; EP 0425661; EP 0589877, EP 0436597; US patents 5,223,409, 5,571,698, 5,885, 793, 5, 969,108, 6,172,197, 6,265,150. The library used for such screening may be generated by using the genetic information of one or more light chains, combined with genetic information encoding a plurality of heavy chains. The library described by De Kruif et al. (1995b)

comprises 7 light chains. Therefore, in a panel of phages binding to a target, which can e.g. be obtained by methods described in De Kruif et al (supra); US patent 6,265,150; not more than 7 different light chains will be represented, and if the panel is large enough, several phages with the same light chain coupled to unrelated heavy chains may be found. Such phages can be used to obtain the nucleic acid molecules useful in the methods according to the present invention.

It is another aspect of the present invention to provide a method for producing a mixture of antibodies to a target, the method comprising the steps of: i) bringing an antibody display library comprising antibodies or antibody fragments into contact with material comprising a target, ii) at least one step of selecting antibodies or antibody fragments binding to said target, iii) identifying at least two antibodies or antibody fragments binding to said target, wherein said at least two antibodies or antibody fragments comprise a common light chain, iv) introducing a nucleic acid sequence encoding the light chain and a nucleic acid sequence or nucleic acid sequences encoding the heavy chains of said at least two antibodies into a host cell, v) culturing a clone of said host cell under conditions conducive to expression of said nucleic acid sequences. The antibody display library may be a phage display library, a ribosome display library, an mRNA display library, or a yeast display library. Step i) and ii) may optionally be repeated one or more times.

The nucleic acid sequences encoding the antibodies obtained by the phage display, ribosome display or yeast display method may be converted to encode any desired antibody format such as IgG1, IgG2, IgG3, IgG4, IgA, IgM, IgD, IgE, before introducing them into a host cell, using standard molecular

cloning methods and means known to the person skilled in the art (e.g. described in Boel et al, 2000).

It will be clear to the skilled person that libraries in which only one light chain is represented are especially useful in light of the present invention, since all antibodies that can be obtained from such a library, will have a common light chain that is functional in binding target antigen with each of the heavy chains. In other words, in accordance with the methods of the invention the formation of non-functional light chain-heavy chain dimers is avoided. Phage antibody display libraries having extensive H chain repertoires and unique or very few L chain sequences have been disclosed in the art (Nissim et al, 1994; Vaughan et al, 1996). In general, the specificity of an antibody appears to be determined to a large extent by its heavy chain, which is supported by the following observations. In the process of receptor editing, a mechanism of B-cells to monitor if their immunoglobulin receptor encodes a potentially harmful autoantibody, B-cells expressing an autoantibody replace the expressed heavy chain with another heavy chain while retaining the the expressed light chain. Thus, a new antibody specificity is generated that does not encode an autoantibody. This shows that a single light chain can successfully dimerize with multiple heavy chains to form different antibody specificities (Nemazee and Weigert, 2000; Nemazee, 2000; Casellas et al, 2001). Series of transfected cell lines using a single heavy chain gene with different light chain genes have been reported, the antibodies produced to a large extent maintaining their specificity, regardless of the light chain (Radic et al, 1991). Different antibodies have been obtained from a library that has been constructed using a single light chain (Nissim et al, 1994). We have

obtained several antibodies from the library described by De Kruif et al (1995), which was constructed using 7 light chains, that have the same light chain but different specificities (see e.g. example 1: antibodies binding to EpCAM and to CD46, described in WO 01/48485 and WO 02/18948, respectively). Besides screening a phage library against a target, it will be also possible to start with an antibody that has already proven its merits, and use the light chain of this antibody in the preparation of a library of heavy chains combined with this particular light chain only, according to methods known to the person skilled in the art (see references supra about phage display). Using this strategy, a monoclonal antibody can be used to obtain a mixture of antibodies according to the invention, functionally resembling a polyclonal or oligoclonal antibody to the same target. Alternatively, a method reminiscent of the method described by Jespers et al (1994) to obtain a human antibody based on a functional rodent antibody can be used. The heavy chain of a known antibody of non-human origin is first cloned and paired as a template chain with a repertoire of human light chains for use in phage display, after which the phages are selected for binding to the antigen or mixture of antigens. The selected light chain is in turn paired with a repertoire of human heavy chains displayed on a phage, and the phages are selected again to find several heavy chains that when paired with the light chain are able to bind to the antigen or mixture of antigens of interest. This enables creating a mixture of human antibodies against a target for which thus far only a non-human monoclonal antibody is described. It is possible that a mixture according to the present invention already has beneficial functional effects when the individual antibodies

do not have high affinities for the target, whereas high affinities are often required for monoclonal antibodies to be effective. This would have the advantage that affinity maturation may be required in less instances for methods and mixtures according to the present invention than when an approach with monoclonal antibodies is envisaged.

The heavy and light chain coding sequences can be introduced simultaneously or consecutively into the host cell. It is also an aspect of the invention to prepare a host cell comprising a recombinant nucleic acid encoding a light chain of an antibody. Such a cell can for instance be obtained by transfection of said nucleic acid, and optionally a clone can be identified that has a high expression of the light chain. An established clone may then be used to add genetic information encoding 2, 3, 4, 5, 6, 7, 8, 9, 10, or more heavy chains of the invention by introducing the nucleic acid molecules encoding these into cells of the clone that already contains the light chain. The nucleic acid molecules encoding the heavy chains may be introduced into said host cell concomitantly. It is of course also possible to introduce them consecutively, e.g. by using different selection markers, which can be advantageous if not all heavy chains can be introduced simultaneously because the cells do not take up enough copies of recombinant nucleic acid molecules. Methods to introduce recombinant nucleic acid molecules into host cells are well known to the person skilled in the art, and include transfection, electroporation, calcium phosphate precipitation, virus infection, and the like. The skilled person has several possibilities to introduce more vectors with nucleic acid sequences of interest into the same host cell, see e.g.

Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edition, 1989; Current Protocols in Molecular Biology, Ausubel FM, et al, eds, 1987; the series Methods in Enzymology (Academic Press, Inc.). Suitable dominant selection markers for introducing nucleic acids into eukaryotic host cells according to the invention may be G418 or neomycin (geneticin), hygromycin or mycophenolic acid, puromycin, and the like, for which genes encoding resistance are available on expression vectors. Further possibilities include for instance the use of vectors containing DHFR genes or glutamate synthetase to select in the presence of methotrexate in a DHFR$^-$ cell or the absence of glutamine in a glutamine auxotroph, respectively. The use of expression vectors with different selection markers enables subsequent transfections with heavy chain sequences of interest into the host cell, which already stably contains other heavy chains introduced previously by use of other selection markers. It is also possible to use selection markers that can be used more than once, e.g. when containing mutations, introns, or weakened promoters that render them concentration dependent (e.g. EP0724639; WO01/32901; US patent 5,733,779). Alternatively, a selection marker may be re-used by deleting it from the host cell after use, e.g. by site-specific recombination. A selectable marker located between sequences recognized by a site-specific recombinase, e.g. lox-sites or FRT-sites, is used for the generation of the first stable transfectant (see for Cre-lox site-specific recombination Wilson and Kola, 2001). Subsequently, the selectable marker is excised from the host cell DNA by the matching site-specific recombinase, e.g. Cre or Flp. A subsequent transfection can suitably use the same selection marker. Different host cell clones each comprising the genetic

information encoding a different light chain may be prepared. If the antibodies are identified by an antibody display method, it is thus possible to prepare several host cells, each comprising one light chain present in the antibody display library. After identifying antibodies that bind to a target using antibody display, the nucleic acid molecules encoding the heavy chains can be introduced into the host cell containing the common light chain that is capable of pairing to the heavy chains. It is therefore an aspect of the present invention to provide a method for making a host cell for production of a mixture of antibodies, the method comprising the steps of: introducing into said host cell a nucleic acid sequence encoding a light chain and nucleic acid sequence or sequences encoding 3, 4, 5, 6, 7, 8, 9, 10, or more different heavy chains that are capable of pairing with said light chain, wherein said nucleic acid molecules are introduced consecutively or simultaneously. It is of course also possible to introduce at least two of said nucleic acid molecules simultaneously, and introduce at least one other of said nucleic acid molecules consecutively. In yet another aspect according to the invention, a method is provided for making a recombinant host cell for production of a mixture of antibodies, the method comprising the step of: introducing a nucleic acid sequence or nucleic acid sequences encoding 2, 3, 4, 5, 6, 7, 8, 9, 10, or more different heavy chains into a recombinant host cell comprising a nucleic acid sequence encoding a light chain capable of pairing with at least two of said heavy chains.

In case it would appear that a recombinant host cell according to the invention does not express sufficient light chain to dimerize with all of the expressed at least two

heavy chains, extra copies of the nucleic acid molecules encoding the light chain may be transfected into the cell.

Besides random integration after transfection, methods to integrate the transgenes in predetermined positions of the genome resulting in favorable expression levels, can also be used according to the invention. Such methods may for instance employ site-specific integration by homologous recombination (see e.g. WO 98/41645), or make use of site-specific recombinases (Gorman and Bullock, 2000).

It is yet another aspect of the present invention to provide a transgenic non-human mammal or a transgenic plant comprising a nucleic acid sequence encoding a light chain and a nucleic acid sequence or nucleic acid sequences encoding at least two different heavy chains that are capable of pairing with said light chain, wherein said nucleic acid sequences encoding said light and heavy chains are under the control of a tissue-specific promoter. Promoters in plants may also be non-tissue specific, and general gene expression elements, such as the CaMV 35S promoter and nopaline synthase polyA addition site can also be used. Said light chain is a common light chain according to the invention. In specific embodiments, the transgenic animal or plant according to the invention comprises 3, 4, 5, 6, 7, 8, 9, 10, or more heavy chain sequences. Besides cell culture as a production system for recombinant proteins, the art also discloses the use of transgenic animals, transgenic plants, and for instance transgenic chickens to produce proteins in the eggs, and the like to produce recombinant proteins of interest (Wilmut et al, 1991; Pollock et al, 1999; Larrick and Thomas, 2001; WO 90/05188; WO 91/08216; WO 92/22644; WO 93/03164; WO 94/05796; WO 93/25567). These usually comprise the recombinant gene or

genes encoding one or more proteins of interest in operable association with a tissue-specific promoter. It has for instance be shown that recombinant antibodies can be produced at high levels in the milk of transgenic animals, that contain the nucleic acids encoding a heavy and a light chain behind a mammary gland specific promoter (e.g. Limonta et al, 1995; Pollock et al, 1999; Van Kuik-Romein et al, 2000; see for extensive description WO 95/17085, US patents 5827690 and 5849992). Particularly useful in this respect are cows, sheep, goats, pigs, rabbits, mice, and the like, which can be milked to obtain antibodies. Useful promoters are the casein promoters, such as the β-casein promoter, the αS1-casein promoter, the whey acidic protein (WAP) promoter, the β-lactoglobulin promoter, the α-lactalbumin promoter, and the like. Production of biopharmaceutical proteins in the milk of transgenic mammals has been extensively described in (Platenburg et al, 1994; Limonta et al, 1995; Pollock et al, 1999; Van Kuik-Romeijn et al, 2000; WO 90/05188; WO 91/08216; WO 92/22644; WO 93/03164; WO 94/05796; WO 93/25567; WO 95/17085). Besides mammary gland specific promoters, also other tissue-specific promoters may be used, directing the expression to the blood, urine, saliva, and the like. The generation of transgenic animals comprising recombinant nucleic acid molecules has been extensively documented, and may include micro-injection of oocytes (see e.g. Wilmut and Clark, 1991; Platenburg et al, 1994), nuclear transfer after transfection (see e.g. Schnieke et al, 1997), infection by recombinant viruses (e.g. Archer et al, 1994; US patent 6291740), and the like. Nuclear transfer and cloning methods for mammalian cells are known to the person skilled in the art, and are e.g. described in (Campbell et al, 1996; Wilmut

et al, 1997; Dinneys et al, 2002; WO 95/17500; WO 98/37183; WO 98/39416; WO 00/31237; WO 00/42174; EP1149898; US patent 6011197). It is nowadays possible to clone most of these animals, to generate lines of animals that are genetically identical, which renders it possible for a person skilled in the art to create such a line once an individual animal producing the desired mixture of antibodies has been identified. Alternatively, classical breeding methods can be used to generate transgenic offspring. Strategies for the generation of transgenic animals for production of recombinant proteins in milk are described in Brink et al, 2000.

Transgenic plants or plant cells producing antibodies have also been described (Hiatt et al, 1989; Larrick et al, 2001; Peeters et al, 2001; Fischer et al, 2000), and useful plants for this purpose include corn, maize, tobacco, soybean, alfalfa, rice, and the like. Constitutive promoters that can for instance be used in plant cells are the CaMV 35S and 19S promoters, Agrobacterium promoters *nos* and *ocs*. Other useful promoters are light inducible promoters such as rbcS. Tissue-specific promoters can for instance be seed-specific, such as promoters from zein, napin, beta-phaseolin, ubiquitin, or tuber-specific, leaf-specific (e.g. useful in tobacco), root-specific, and the like. It is also possible to transform the plastid organelle by homologous recombination, to express proteins in plants. Methods and means for expression of proteins in recombinant plants or parts thereof, or recombinant plant cell culture, are known to the person skilled in the art and have been for instance been described in (Giddings et al, 2000; WO 01/64929; WO 97/42313; US patents 5202422, 5888789, 5959177, 6080560; See for practical guidelines: Methods In Molecular Biology vol. 49

"Plant Gene Transfer And Expression Protocols", Jones H, 1995). Other transgenic systems for producing recombinant proteins have also been described, including the use of transgenic birds to produce recombinant proteins in eggs (e.g WO 97/47739), and the use of transgenic fish (e.g. WO 98/15627), and can be used in combination with the teachings of the present invention to obtain mixtures of antibodies. It is also possible to use an in vitro transciption/translation or in vitro translation system for the expression of mixtures of antibodies according to the present invention. It will be clear to the skilled person that the teachings of the current invention will allow producing mixtures of antibodies in systems where recombinant nucleic acid encoding the light chain and heavy chains can be introduced and expressed. Preferably such systems are able to produce antibodies encoded by said nucleic acid sequences, without the use of amplification of said nucleic acid sequences in said systems. In another aspect of the invention, a cell from a transgenic non-human animal or a transgenic plant according to the invention is provided. Such cells can be used to generate the animals or plants according to the invention, using techniques known to the person skilled in the art, such as nuclear transfer or other known methods of cloning whole organisms from single cells. The cells according to the invention may also be obtained by introducing the light and at least two heavy chain sequences into isolated cells of non-human animals or plants, which cells are capable of becoming part of a transgenic animal or plant. Particularly useful for such purposes are embryonic stem cells. These can contribute to the germ line, and therefore the genetic information introduced into such cells can be passed to future generations. In addition, plant cell cultures of

cotton, corn, tomato, soybean, potato, petunia, and tobacco can be utilized as hosts, when transformed with the nucleic acid molecules encoding the light chain and the heavy chains, e.g. by use of the plant transforming bacterium Agrobacterium tumefaciens or by particle bombardment, or by infecting with recombinant plant viruses.

It is another aspect of the present invention to provide a pharmaceutical composition comprising a mixture of recombinantly produced antibodies and a suitable carrier, wherein at least two different heavy chains are represented in said mixture of recombinantly produced antibodies. Pharmaceutically acceptable carriers as used herein are exemplified, but not limited to, adjuvants, solid carriers, water, buffers, or other carriers used in the art to hold therapeutic components, or combinations thereof. In particular embodiments, 3, 4, 5, 6, 7, 8, 9, 10, or more different heavy chains are represented in said mixture. Said mixture can be obtained by mixing recombinantly produced monoclonal antibodies, but may also be obtained by methods according to the present invention. Said mixture may therefore comprise a common light chain for said antibodies. Said mixture may comprise bispecific antibodies. Said mixture may be produced from a clone that was derived from a single host cell, i.e. from a population of cells containing the same recombinant nucleic acid molecules. The term "recombinantly produced" as used herein refers to production by host cells that produce antibodies encoded by recombinant nucleic acids introduced in such host cells or ancestors thereof. It does therefore not include the classical method of producing polyclonal antibodies, whereby a subject is immunized with an antigen or antigen comprising mixture,

after which the antibodies produced by this subject are recovered from the subject, e.g. from the blood.

It is another aspect of the present invention to provide a mixture of antibodies wherein at least two heavy chains are represented, for use in the treatment or diagnosis of a human or animal subject. In another aspect, the invention provides the use of a mixture of antibodies wherein at least two different heavy chains are represented, for the preparation of a medicament for use in the treatment or diagnosis of a disease or disorder in a human or animal subject. In particular embodiments, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more heavy chains are represented in said mixture. Said mixtures of antibodies may be mixtures of antibodies according to the invention, or obtained by methods according to the invention. Antibodies present in said mixture may preferably comprise a common light chain. The mixtures may comprise bispecific antibodies, and may be recombinantly produced from a clone that was derived from a single host cell, i.e. from a population of cells containing the same recombinant nucleic acid molecules. The targets may be used to screen an antibody display library, as described supra, to obtain 2, 3, 4, 5, 6, 7, 8, 9, 10, or more antibodies comprising a common light chain that bind to the target, and produce a mixture of these according to the teachings of the present invention. Virtually any area of medicine where monoclonal antibodies can be used is amenable for the use of the mixtures according to the invention. This can e.g. include treatment of auto-immune diseases and cancer, including solid tumors of the brain, head- and neck, breast, prostate, colon, lung, and the like, as well as hematologic tumors such as B-cell tumors. Neoplastic disorders which can be treated with the mixtures

according to the present invention include leukemias, lymphomas, sarcomas, carcinomas, neural cell tumors, squamous cell carcinomas, germ cell tumors, metastases, undifferentiated tumors, seminomas, melanomas, myelomas, neuroblastomas, mixed cell tumors, neoplasias caused by infectious agents, and other malignancies. Targets for the antibody mixtures may include, but are not limited to, the HER-2/Neu receptor, other growth factor receptors such as VEGFR1 and VEGFR2 receptor, B-cell markers such as CD19, CD20, CD22, CD37, CD72, etc, T-cell markers such as CD3, CD25, etc, other leukocyte cell surface markers such as CD33 or HLA-DR, etc, cytokines such as TNF, interleukins, receptors for these cytokines such as members of the TNF receptor family, and the like. It is anticipated that the use of such mixtures of antibodies in the treatment of cancerous tissues or other complex multi-antigen comprising cells such as microorganisms or viruses will give rise to less occurrence of epitope-loss escape variants than the use of single monoclonal antibodies. Several treatments nowadays use polyclonal mixtures of antibodies, which are derived from immunized humans or animals. These treatments may be replaced by use of the mixtures according to the present invention. Use of these mixtures can also include use in graft-versus-host rejections, known in the art of transplantation, e.g. by use of anti-thymocyte antibodies. It is anticipated that the mixtures of antibodies are superior to monoclonal antibodies in the treatment of complex antigens or antigen comprising mixtures such as bacteria or viruses. Therefore, use according to the invention can also include use against strains of bacteria and fungi, e.g. in the treatment of infectious diseases due to pathogenic bacteria such as multidrug resistant S.aureus and the like, fungi such as

Candida albicans and Aspergillus species, yeast and the like. The mixtures according to the invention may also be used for post exposure profylaxis against viruses, such as members of the genus Lyssavirus e.g. rabies virus, or for therapeutic use against viruses such as Varicella-Zoster Virus, Adenoviruses, Respiratory Syncitium Virus, Human Immunodeficiency Virus, Human Metapneumovirus, Influenzavirus, and the like. Mixtures according to the inventions can also be used to protect against agents, both bacteria and viruses, and against toxic substances that are potential threats of biological warfare. Therefore, use according to the invention can also include use against strains of bacteria such as *Bacillus anthracis, Clostridium botulinum* toxin, *Clostridium perfringens* epsilon toxin *Yersinia Pestis, Francisella tulariensis, Coxiella burnetii, Brucella* species, *Staphylococcus* enterotoxin B, or against viruses such as Variola major, alphaviruses causing meningoencephalitis syndromes (EEEV, VEEV, and WEEV), viruses known to cause hemorrhagic fevers such as Ebola, Marburg and Junin virus or against viruses such as Nipah virus, Hantaviruses, Tickborne encephalitis virus and Yellow fever virus or against toxins e.g. Ricin toxin from Ricinus communis and the like. Use of the mixtures according to the invention can also include use against unicellular or multicellular parasites. Recombinant mixtures of antibodies according to the invention may become a safe alternative to polyclonal antibodies obtained from pools of human sera for passive immunization, or from sera of hyper-immunized animals. The mixtures may be more efficacious in various therapeutic applications than recombinant monoclonal antibodies, including cancer, allergy, viral diseases, chronic inflammation, and the like.

It has been described that homodimerization of tumor-reactive monoclonal antibodies markedly increases their ability to induce growth arrest or apoptosis of tumor cells (Ghetie et al, 1997). Possibly, when antibodies against receptors or other surface antigens on target cells, such as tumor cells or infectious microorganisms, are produced according to the present invention, the bispecific antibodies present in mixtures according to the invention may also crosslink different receptors or other antigens on the surface of target cells, and therefore such mixtures may be very suitable for killing such cells. Alternatively, when bispecific antibodies are less desirable, the present invention also provides methods to recombinantly produce mixtures of antibodies comprising mainly monospecific antibodies. It has been described that the efficacy of treatment with Rituximab™ (anti-CD20 monoclonal antibody) was increased when anti-CD59 antibodies were added (Herjunpaa et al, 2000). Therefore, it is expected that inclusion of antibodies against CD59 in a mixture according to the invention comprising anti-tumor antibodies in the form of B-cell receptor recognizing antibodies increases the sensitivity of tumor cells to complement attack. It has also been shown that a triple combination cocktail of anti-CD19, anti-CD22, and anti-CD38-saporin immunotoxins is much more effective than the individual components in the treatment of human B-cell lymphoma in an immunodeficient mouse model (Flavell et al, 1997). Many other combinations may also be feasible and can be designed by the person skilled in the art. In general, the use of antibody mixtures that are capable of recognizing multiple B-cell epitopes will likely decrease the occurrence of escape variants.

Another possible target is a transmembrane tyrosine kinase

receptor, encoded by the Her-2/Neu (ErbB2) proto-oncogene (see e.g. US patents 5,772,997 and 5,783,186 for anti-Her2 antibodies). Her-2 is overexpressed on 30% of highly malignant breast cancers, and successful antibodies against this target, marketed under the name Herceptin™ (Trastuzumab), have been developed. It has been shown that targeting multiple Her-2 epitopes with a mixture of monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line in vitro and in vivo (Spiridon et al, 2002). Her-2 may therefore be a good target for antibody mixtures according to the present invention. Antibodies useful for this purpose can be obtained by methods described in the present invention, including antibody display methods.

Human antibodies are capable of eliciting effector function via binding to immunoglobulin receptors on immune effector cells. Human IgG, and in particular IgG_1 and IgG_3, fix complement to induce CDC and interact with Fcγ receptors to induce antibody dependent cell mediated cytotoxicity (ADCC), phagocytosis, endocytosis, induction of respiratory burst and release of inflammatory mediators and cytokines. Human IgA interacts with FcαR, also to result in efficient activation of ADCC and phagocytosis of target cells. Hence, due to the differential distribution of FcγR and FcαR on peripheral blood cells (Huls et al., 1999), using a mixture of antibodies directed against the target and consisting of both IgG and IgA would potentially maximize the recruitment and activation of different immune effector cells. Such a mixture of both IgG and IgA could be obtained by producing the IgG and IgA monoclonal antibody in a separate production process

using two distinct production cell lines, but could also be obtained from a single cell line producing both the IgG and the IgA monoclonal antibody. This would have the advantage that only a single production process has to be developed. Thus when different heavy chains are mentioned, also heavy chains differing in their constant regions are encompassed in the invention. The principle of using a common light chain can also be used for the production of a mixture of isotypes from a host cell. It is therefore yet another aspect of the present invention to provide a method for producing a mixture of antibodies comprising different isotypes from a host cell, the method comprising the step of: culturing a host cell comprising a nucleic acid sequence encoding a light chain and nucleic acid sequences encoding at least two heavy chains of different isotype that are capable of pairing with said light chain, under conditions conducive to expression of said nucleic acid sequences. According to this aspect of the invention, different heavy chains may have identical variable regions, and only differ in their constant regions (i.e. be of different isotype and have the same specificity). In a particular embodiment, said isotypes comprise at least an IgG and an IgA and/or IgM, preferably IgG1 or IgG3 and IgA. Other combinations of IgG1, IgG2, IgG3 and IgG4 can also be used.

In other embodiments according to this aspect of the invention, not only the constant regions of the heavy chains may differ, but also the variable regions, thereby giving rise to different specificities, paired with the same light chain. When bispecific antibodies are not desired for a given purpose, e.g. because the mixtures of antibodies are less efficacious because of the presence of the bispecific antibodies, it is possible to use at least two heavy chains combined with the common light chain according to the

invention wherein said heavy chains differ sufficient in their constant regions to reduce or prevent pairing between the different heavy chains, e.g. by using heavy chains of different isotypes, e.g. an IgG1 and an IgG3. It is anticipated that the heavy chains of different isotype will pair much less efficient, if at all, compared to the same heavy chains. Alternatively, it is also possible to engineer the different heavy chains in their constant region such that homodimerization is favored over heterodimerization, e.g. by introducing self-complementary interactions (see e.g. WO 98/50431 for possibilities, such as "protuberance-into-cavity" strategies (see WO 96/27011)). It is therefore another aspect of the present invention to provide a method for producing a mixture of antibodies in a recombinant host, the method including the step of: expressing in a recombinant host cell a nucleic acid sequence encoding a common light chain and nucleic acid sequences encoding at least two different heavy chains that differ in the variable region and that are capable of pairing with said common light chain, and wherein said heavy chains further differ in their constant regions sufficiently to reduce or prevent pairing between the different heavy chains. In one embodiment, said heavy chains are of different isotype. In specific embodiments, 3, 4, 5, 6, 7, 8, 9, 10, or more different heavy chains are expressed. Mixtures of antibodies obtainable by this method are also embodied in the present invention. Such mixtures will comprise mainly monospecific antibodies.

The teachings of the present invention can also be used to obtain novel multispecific antibodies or mixtures thereof. Therefore, in another aspect, the invention provides a method for producing a mixture of antibodies comprising dimeric IgA isotype {$(IgA)_2$} antibodies in a recombinant host, wherein at

least part of said dimeric IgA antibodies have different binding regions in each of the IgA subunits, the method comprising the step of: expressing in a recombinant host cell a nucleic acid sequence encoding a common light chain and nucleic acid sequences encoding at least two different heavy chains of IgA isotype capable of pairing to said common light chain, wherein said different heavy chains differ in their variable region. Dimerization of the IgA molecules can be enhanced by co-expressing J-chain (Yoo et al, 1999). Said dimeric IgA antibodies have two specificities (see Fig.10 for a schematic representation of one possible form produced and present in the mixture). In yet another aspect the invention provides a method for producing a mixture of antibodies comprising an IgM antibody having at least two different specificities, the method comprising the step of expressing in a recombinant host cell a nucleic acid sequence encoding a common light chain and nucleic acid sequences encoding at least two different heavy chains of IgM isotype, wherein said heavy chains are capable of pairing to said common light chain and form functional antigen binding regions. Up to five specificities can be comprised in an IgM pentamer in the presence of J-chain, and up to six in an IgM hexamer in the absence of J-chain (Yoo et al, 1999). Therefore, in specific embodiments, 3, 4, 5, or 6 IgM heavy chains are co-expressed with the common light chain according to this aspect of the invention. See Fig. 11 for a schematic representation of one of the possible forms that can be produced and present in the mixture according to this aspect of the invention, when five different heavy chains are expressed with a common light chain. The invention also provides for IgA dimers, IgM pentamers or hexamers having at least two different specificities. These molecules can be produced from a clone

of a single host cell according to the invention. Such molecules, harbouring antigen binding regions with different specificities, can bind different epitopes on the same antigen, different antigens on one cell, or different antigens on different cells, thereby crosslinking the antigens or cells.

It is yet another aspect of the present invention to provide a method for identifying a mixture of antibodies having a desired effect in a functional assay, the method comprising the steps of i) adding a mixture of antibodies in a functional assay, and ii) determining the effect of said mixture in said assay, wherein said mixture of antibodies comprises antibodies having a common light chain. In a preferred embodiment said mixture is comprised in a composition according to the present invention.

Examples

The following examples are provided to illustrate the invention, and are not to be construed in any way to limit the scope of the invention. The practice of this invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, microbiology, cell biology, and recombinant DNA, which are within the skill of the art. See e.g. Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edition, 1989; Current Protocols in Molecular Biology, Ausubel FM, et al, eds, 1987; the series Methods in Enzymology (Academic Press, Inc.); PCR2: A Practical Approach, MacPherson MJ, Hams BD, Taylor GR, eds, 1995; Antibodies: A Laboratory Manual, Harlow and Lane, eds, 1988.

Example 1 Production of a mixture of monoclonal antibodies with a common light chain and two heavy chains in a single cell

Clone UBS-54 and Clone K53 were previously isolated by selections on the colorectal cell line SW40 (Huls et al., 1999) and on a heterogeneous mixture of mononuclear cells of a patient with multiple myeloma (WO 02/18948), respectively, with a semi-synthetic library (de Kruif et al., 1995b). Further studies revealed that clone UBS-54 and K53 bound to the EP-CAM homotypic adhesion molecule (Huls et al., 1999) and the membrane cofactor protein CD46 (WO 02/18948), respectively. DNA sequencing of the clones revealed that they were unique in the Heavy chain CDRs, but that they contained an identical light chain sequence (Fig. 3). The V_H and V_L of clones UBS-54 and K53 were inserted into an expression vector

containing the HAVT20 leader sequence and all the coding sequences for the constant domains of a human IgG1 with a Kappa light chain by a method essentially as described (Boel et al, 2000), which resulted in plasmids pUBS3000Neo and pCD46_3000(Neo) (Fig. 4). These plasmids were transiently expressed either alone or in combination in PER.C6™ cells. In brief, each 80 cm^2 flask was transfected by incubation for 4 hours with 140 µl lipofectamine + 10 µg DNA (either pUBS3000Neo, pCD46_3000(Neo) or 10 µg of both) in serum-free DMEM medium at 37°C. After 4 hours, this was replaced with DMEM + 10% FBS, and the cells were grown overnight at 37°C. Cells were then washed with PBS and the medium was replaced with Excell 525 medium (JRH Bioscience). The cells were allowed to grow at 37°C for 6 days, after which the cell culture supernatant was harvested. Human IgG specific ELISA analysis (described in WO 00/63403) indicated that IgG was present at approximately 10 µg/ml for all flasks containing expression plasmids. No IgG1 was present in a control flask which was not transfected with expression plasmid.
Human IgG from each supernatant was subsequently purified using Protein A affinity chromatography (Hightrap Protein A HP, cat.no. 1-040203) according to standard procedures, following recommendations of the manufacturer (Amersham Biosciences). After elution, samples were concentrated in a Microcon YM30 concentrator (Amicon) and buffer exchanged to 10 mM sodium phosphate, pH 6.7. Twelve µg of purified IgG was subsequently analyzed on Isoelectric focusing gels (Serva Pre-cast IEF gels, pH range 3-10, cat.no. 42866). The samples were loaded on the low pH side and after focussing stained with colloidal blue (Fig. 5). Lane 1 shows transiently expressed K53, Lane 2 shows transiently expressed UBS-54 and Lane 3 shows the IgG sample of the cells in which both

antibodies were co-transfected. Clearly, K53 and UBS-54 each have a unique pI profile and the sample form the cotransfection showed other unique isoforms, with the major isoform having a pI in between those of K53 and UBS-54. This is also anticipated on the basis of the theoretic pI when calculated with the ProtParam tool provided on the Expasy homepage (http://www.expasy.ch; Appel et al., 1994). K53 and UBS-54 have a theoretic pI of 8.24 and 7.65, respectively, whereas an isoform representing a heterodimer of one UBS-54 heavy chain and one K53 heavy chain has a theoretical pI of 8.01. Assembly of such a heterodimer can only occur when a single cell translates both the heavy chain of K53 and the heavy chain of UBS-54 and assembles these into a full length IgG molecule together with the common light chain. Therefore, this experiment shows that it is possible to express two unique human IgG molecules in a single cell and that a heterodimer consisting of these two unique binding specificities is also efficiently formed. To investigate whether more complex mixtures according to the invention can be prepared, analogously to this example, the experiment is performed expressing three different heavy chains and one light chain.

Example 2 Production of a mixture of antibodies against human B-cell markers in a clonal PER.C6™ cell line.

A method for producing a mixture of antibodies according to the invention, using expression in a recombinant host cell of a single light chain and three different heavy chains capable of pairing to the single light chain to form functional antibodies, is exemplified herein and is schematically shown in Fig. 6.

Phages encoding antibodies capable of binding proteins present on human B-cells, i.e. CD22, CD72 and Major Histocompatibility Complex (MHC)class II (further referred to as HLA-DR) were previously isolated from a semi-synthetic phage library (de Kruif et al., 1995; van der Vuurst de Vries & Logtenberg, 1999). DNA sequencing of the V_H and V_L sequences of the phages clone B28 (anti-CD22), clone I-2 (anti-HLA-DR) and clone II-2 (anti-CD72) revealed that they all contain a unique V_H sequence, but a common light chain sequence (Vλ3) with an identical CDR region (Fig. 7).

The V_H and V_L sequences of clones B28, I-1 and II-2 are cloned behind the HAVT20 leader sequences of expression plasmid pCRU-L01 (Fig. 8), or a plasmid derived from pCRU-L01, to obtain plasmids encoding a full length human IgG_1-lambda with binding specificities for CD22, CD72 and HLA-DR. These plasmids are designated pgG102-120-C01, pgG102-025-C01, and pgG102-026-C01 respectively, but will further be referred to as pCRU-CD22, pCRU-CD72 and pCRU-HLA-DR, respectively.

Stable PER.C6™ derived cell lines are generated, according to methods known to the person skilled in the art (see e.g. WO 00/63403), the cell lines expressing antibodies encoded by genetic information on either pCRU-CD22, pCRU-CD72 or pCRU-HLA-DR and a cell line expressing antibodies encoded by all three plasmids. Therefore, PER.C6™ cells are seeded in DMEM

plus 10% FBS in tissue culture dishes (10 cm diameter) or T80 flasks with approximately 2.5 x 10^6 cells per dish and kept overnight under their normal culture conditions (10% CO_2 concentration and 37°C). The next day, transfections are performed in separate dishes at 37°C using Lipofectamine (Invitrogen Life Technologies) according to standard protocols provided by the manufacturer, with either 1-2 µg pCRU-CD22, 1-2 µg pCRU-CD72, 1-2 µg pCRU-HLA-DR or 1 µg of a mixture of pCRU-CD22, pCRU-CD72 and pCRU-HLA-DR. As a control for transfection efficiency, a few dishes are transfected with a LacZ control vector, while a few dishes will be not transfected and serve as negative controls.

After 4 to 5 hours, cells are washed twice with DMEM and refed with fresh medium without selection. The next day, medium are replaced with fresh medium containing 500 µg/ml G418. Cells are refreshed every 2 or 3 days with medium containing the same concentrations of G418. About 20-22 days after seeding, a large number of colonies are visible and from each transfection at least 300 are picked and grown via 96-well and/or 24-well via 6-well plates to T25 flasks. At this stage, cells are frozen (at least 1, but usually 4 vials per sub-cultured colony) and production levels of recombinant human IgG antibody are determined in the supernatant using an ELISA specific for human IgG_1 (described in WO 00/63403). Also, at this stage G418 is removed from the culture medium and never re-applied again. For a representative number of colonies larger volumes will be cultured to purify the recombinant human IgG_1 fraction from the conditioned supernatant using Protein A affinity chromatography according to standard procedures. Purified human IgG_1 from the various clones is analyzed on SDS-PAGE, Iso-electric focusing (IEF) and binding to the targets CD22, CD72 and HLA-DR using cell

transfectants expressing these human antigens on their cell surface (transfectants expressing CD72 and HLA-DR have been described by van der Vuurst-de Vries and Logtenberg, 1999; a CD22 transfectant has been prepared according to similar standard procedures in PER.C6™).

Colonies obtained from the co-transfection with pCRU-CD22, pCRU-CD72 and pCRU-HLA-DR are screened by PCR on genomic DNA for the presence or absence of each of the three constructs. The identity of the PCR products is further confirmed by DNA sequencing.

Next, it is demonstrated that a clonal cell line accounts for the production of each of the three binding specificities, i.e. proving that a single cell is able to produce a mixture of more than two functional human IgG's. Therefore, a limited number of colonies, which screened positive for the production of each of the three binding specificities (both by PCR at the DNA level as well as in the specified binding assays against CD22, CD72 and HLA-DR), are subjected to single cell sorting using a fluorescence activated cell sorter (FACS) (Becton & Dickinson FACS VANTAGE SE). Alternatively, colonies are seeded at 0.3 cells/well to guarantee clonal outgrowth. Clonal cell populations, hereafter designated as sub-clones, are refreshed once a week with fresh medium. Sub-clones are grown and transferred from 96-wells via 24- and 6-wells plates to T25 flasks. At this stage, sub-clones are frozen (at least 1, but usually 4 vials per sub-clone) and production levels of recombinant human IgG_1 antibody are determined in the supernatant using a human IgG_1 specific ELISA. For a representative number of sub-clones, larger volumes are cultured to purify the recombinant human IgG_1 fraction from the conditioned supernatant using

Protein A affinity chromatography according to standard procedures.

Purified human IgG_1 from the various sub-clones is subsequently analyzed as described above for human IgG_1 obtained from the parental clones, i.e. by SDS-PAGE, Iso-electric focusing (IEF) and binding to the targets CD22, CD72 and HLA-DR. Sub-clones will also be screened by PCR on genomic DNA for the presence or absence of each of the three constructs pCRU-CD22, pCRU-CD72 and pCRU-HLA-DR. The identity of the PCR products is further confirmed by DNA sequencing. Other methods such as Southern blot and/or FISH can also be used to determine whether each of the three constructs are present in the clonal cell line.

Sub-clones that are proven to be transgenic for each of the three constructs are brought into culture for an extensive period to determine whether the presence of the transgenes is stable and whether expression of the antibody mixture remains the same, not only in terms of expression levels, but also for the ratio between the various antibody isoforms that are secreted from the cell. Therefore, the sub-clone culture is maintained for at least 25 population doubling times either as an adherent culture or as a suspension culture. At every 4-6 population doublings, a specific production test is performed using the human IgG specific ELISA and larger volumes are cultured to obtain the cell pellet and the supernatant. The cell pellet is used to assess the presence of the three constructs in the genomic DNA, either via PCR, Southern blot and/or FISH. The supernatant is used to purify the recombinant human IgG_1 fraction as described supra.

Purified human IgG_1 obtained at the various population doublings is analyzed as described, i.e. by SDS-PAGE, Iso-electric focusing (IEF) and binding to the targets CD22, CD72

and HLA-DR using cell transfectants expressing these antigens.

Example 3 Screening of clones expressing multiple human IgGs for the most potent mixture of functional human IgGs

Functionality of the antibody mixture is analyzed in cell-based assays to determine whether the human IgG_1 mixture inhibits proliferation and/or induces apoptosis of B-cell lines, such as for example Ramos. Other cell lines can also be used. In addition the antibody mixtures are analyzed for their potential to induce antibody dependent cellular toxicity and complement dependent cytotoxicity of for example Ramos cells.

In each of the following experiments the functionality of the antibody mixture recognizing the targets CD22, CD72 and HLA-DR is analyzed and can be compared to each of the individual IgG1 antibodies and to an equimolar combination of the three individual IgG1 specificities.

To assess the ability of the antibody mixtures to inhibit the proliferation of Ramos cells, these cells are incubated in 96-well plates ($0.1 - 1.0 \times 10^5$/ml) with several concentrations (5 - 20 µg/ml) of the antibody mixtures against CD22, CD72 and HLA-DR for 24 hours. The proliferation of the cells is measured by ^3H-thymidine incorporation during another 16 hours of culture. Inhibition of growth is determined by plotting the percentage of ^3H-thymidine incorporation compared to untreated cells (taken as 100% reference value).

To analyze apoptosis induction of Ramos cells, these cells are stimulated in 48-well plates ($0.2 - 1.0 \times 10^6$/ml) with several concentrations (5 - 20 µg/ml) of the antibody mixtures against the targets CD22, CD72 and HLA-DR for 24 or 48 hours. After the incubation period the phosphatidyl serine

exposure on apoptotic cells is analyzed (Koopman G et al, 1994). Therefore, the cells are harvested, washed twice with PBS and are incubated at RT for 10 min with 100 µl FITC-labelled annexin V (Caltag) diluted 1:25 in annexin V binding buffer (Caltag). Prior to the analysis of the samples by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA) propidium iodide (PI)(Sigma) is added to a final concentration of 5 µg/ml to distinguish necrotic cells (annexin V-/PI+) from apoptotic cells (annexin V+/PI-, early apoptotic cells; annexin V+/PI+, late apoptotic cells).
In an alternative assay, apoptosis is induced by crosslinking the antibody mixtures against CD22, CD72 and HLA-DR on the cell surface of Ramos cells with 25 µg/ml of F(ab)2 of goat-anti-human (Fc-specific) polyclonal antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) during the incubation period.
In another alternative assay, apoptosis is induced by incubating the Ramos cells with several concentrations (5 - 20 µg/ml) of the antibody mixtures against CD22, CD72 and HLA-DR while co-incubating them with the chemosensitizing agents doxorubicin (Calbiochem) or dexamethasone (UMCU,Utrecht, the Netherlands).

Antibody Dependent Cellular Cytotoxicity of the antibody mixtures is analyzed using peripheral blood mononuclear cells as effector cells in a standard ^{51}Cr release assay (Huls et al, 1999). To this purpose, 1-3 x 10^6 Ramos cells are labelled with 100 µCi (Amersham, Buckinghamshire, UK) for 1 hour at 37°C. After three washes with medium, the Ramos target cells are plated in U bottom 96 well plates at 5 x 10^3 cells/well. Peripheral blood mononuclear cells that are

obtained from healthy donors by Ficoll-Hypaque density gradients are then added to each well at effector:target ratios ranging from 80:1 to 10:1 in triplicate. The cells are incubated at 37°C in the presence of various concentrations of the antibody mixtures (5 - 20 μg/ml) in a final volume of 200 μl. After 4 hours of incubation part of the supernatant is harvested and ^{51}Cr release is measured. The percantage of specific lysis is calculated using the following formula: % specific lysis = ([experimental cpm - spontaneous cpm]/[maximal cpm - spontaneous cpm] x 100%). Maximal ^{51}Cr release is determined by adding triton X-100 to a final concentration of 1% to the target cells and spontaneous release is determined after incubation of the target cells with medium alone.

Complement dependent cytotoxicity is determined in a similar assay. Instead of the effector cells, now 50 μl human serum is added to the target cells. Subsequently, the assay is performed in the same manner.

Alternatively, ADCC and CDC of the antibody mixtures is determined using a Europium release assay (Patel and Boyd, 1995) or using an LDH release assay (Shields et al, 2001).

Example 4 Use of phage display to isolate multiple phages with an identical V_L sequence against a predefined target (Her-2), and production in a recombinant host cell of a mixture of antibodies capable of binding this target.

Phages displaying scFv fragments capable of binding multiple epitopes present on the same protein, for example the epidermal growth factor receptor Her-2, can be isolated from a semi-synthetic phage library (de Kruif et al., 1995a,b). It is possible to identify several of such phages and select the ones comprising the same light chain sequence, for further use according to the invention. The semi-synthetic library is formed by mixing 7 sub-libraries that each contain a different light chain (de Kruif et al, 1995a,b). It is therefore particularly practical to use such a sub-library, containing only one light chain and many heavy chains, for screening so that multiple antibodies with an identical V_L sequence are obtained, and further used for expressing the antibody mixtures according to the invention.

For the selection of phages against Her-2 several fusion proteins are generated comprising different parts of the extracellular domain of Her-2 that are fused to the CH2 and CH3 domains of human IgG1. For this purpose a pCDNA3.1zeo expression vector (InVitrogen) has been constructed that contains in its multiple cloning region an XhoI restriction site in the hinge region in frame prior to the CH2 and CH3 domains of human IgG1. Using a Her-2 cDNA clone as a template PCR fragments is generated using standard molecular biology techniques known to a person skilled in the art. These fragments consist of a unique 5′ restriction site, a start codon followed by a eukaryotic leader sequence that is linked in frame to either the total extracellular (EC) domain of Her-2 or to a part of the EC domain of Her-2 that is followed

in frame by an XhoI restriction site. These PCR fragments are subsequently cloned in frame with the CH2-CH3 IgG1 region into the pCDNA3.1zeo expression vector. In addition to the fusion protein containing the total EC domain of Her-2, several smaller fusion proteins are generated containing non-overlapping fragments of the Her-2 EC domain. These constructs encoding the Her-2-Ig fusionproteins are used for transient transfection of 293T cells using the lipofectamine reagent (Gibco). Five days after transfection the supernatants of the 293T cells are harvested and Her-2-Ig fusion proteins are purified using protein A affinity chromatography according to standard procedures.

Her-2-Ig fusion proteins containing non-overlapping fragments of the Her-2 EC domain are coated for 2 hours at 37° onto the surface of Maxisorp™ plastic tubes (Nunc) at a saturating concentration (0.5 - 5 µg/ml). The tubes are blocked for 1 h in 2% fat free milk powder dissolved in PBS (MPBS). Simultaneously, 500 µl (approximately 10^{13} cfu) of a semi-synthetic phage display library (a sub-library according to the terminology used above) in which only one Vkappa1 light chain is represented prepared as described by De Kruif et al (1995a,b) and references therein, is added to two volumes of 4% MPBS. In addition, human serum is added to a final concentration of 15% and blocking is allowed to proceed for 30-60 min. The Her-2-Ig-coated tubes are emptied and the blocked phage library is added. The tube is sealed and rotated slowly for 1 h, followed by 2 h of incubation without rotation. The tubes are emptied and washed 10 times in PBS containing 0.1% Tween-20, followed by washing 5 times in PBS. 1 ml glycine-HCL, 0.05 M, pH 2.2 is added, and the tube is rotated slowly for 10 min. The eluted phages are added to 500 µl 1M Tris-HCl pH 7.4. To this mixture, 3.5 ml of

exponentially growing XL-1 blue bacterial culture is added. The tubes are incubated for 30 min at 37°C without shaking. Subsequently, the bacteria are plated on 2TY agar plates containing ampicillin, tetracycline and glucose. After overnight incubation of the plates at 37°C, the colonies are scraped from the plates and used to prepare an enriched phage library, essentially as described by De Kruif et al. (1995a). Briefly, scraped bacteria are used to inoculate 2TY medium containing ampicillin, tetracycline and glucose and are grown at 37°C to an OD_{600nm} of ~0.3. Helper phages are added and allowed to infect the bacteria after which the medium is changed to 2TY containing ampicillin, tetracycline and kanamycin. Incubation is continued overnight at 30°C. The next day, the bacteria are removed from the 2TY medium by centrifugation after which the phages are precipitated using polyethylene glycol 6000/NaCl. Finally, the phages are dissolved in a small volume of PBS-1% BSA, filter-sterilized and used for a next round of selection. The selection/re-infection procedure is performed twice. After the second round of selection, individual *E.coli* colonies are used to prepare monoclonal phage antibodies. Essentially, individual colonies are grown to log-phase and infected with helper phages after which phage antibody production is allowed to proceed overnight. Phage antibody containing supernatants are tested in ELISA for binding activity to Her-2-total EC-Ig coated 96 wells plates.

Selected phage antibodies that are obtained in the screen described above, are validated by ELISA for specificity. For this purpose, Her-2-Ig fusion proteins containing non-overlapping fragments of the Her-2 EC domain are coated to Maxisorp ELISA plates. After coating, the plates are blocked

in 2% MPBS. The selected phage antibodies are incubated in an equal volume of 4% MPBS. The plates are emptied, washed once in PBS, after which the blocked phages are added. Incubation is allowed to proceed for 1 h, the plates are washed in PBS 0.1% Tween-20 and bound phages are detected using an anti-M13 antibody conjugated to peroxidase. The procedure is performed simultaneously using a control phage antibody directed against thyroglobulin (De Kruif et al. 1995a,b), which serves as a negative control.

In another assay the selected phage antibodies are analyzed for their ability to bind BT474 human breast cancer cells that express Her-2. For flow cytometry analysis, phage antibodies are first blocked in an equal volume of 4% MPBS for 15 min at 4°C prior to the staining of the BT474 cells. The binding of the phage antibodies to the cells is visualized using a biotinylated anti-M13 antibody (Santa Cruz Biotechnology) followed by streptavidin-phycoerythrin (Caltag).

Alternatively, phage antibodies recognizing multiple epitopes on Her-2 are selected using a method based upon competition of phage binding to Her-2 with binding of the well characterized murine anti-Her-2 antibodies HER50, HER66 and HER70 (Spiridon and Vitetta etal, 2002). To this purpose $2x10^6$ BT474 cells are incubated at 4°C with approximately 10^{13} cfu (0.5 ml) of a semi-synthetic phage display library in which only one Vkappa1 light chain is represented prepared as described supra and blocked with 2 volumes of medium containing 10% of FBS. The mixture is slowly rotated at 4°C for 2 hours in a sealed tube. Subsequently, non-bound phages are removed by two washes with 50 ml of cold medium containing 10% FBS. Hereafter, phages

recognizing multiple epitopes on Her-2 are eluted by resuspending the BT474 cells in 1 ml of cold medium containing saturating concentrations (5-20 μg/ml) of the HER50, HER66 and HER70 murine anti-Her-2 antibodies. The cells are left on ice for 10 min, spun down and the supernatant containing the anti-Her-2 phage antibodies is used to reinfect XL1-Blue cells as described supra.

From the panel of Her-2-specific phage antibodies generated by de screens described above, three phage antibodies are selected that are recognizing three different non-overlapping epitopes on the Her-2 protein.
The V_H sequences and the unique Vkappa1 light chain sequence of these clones, provisionally designated VK1HER2-1, VK1HER2-2 and VK1HER2-3, are cloned behind the HAVT20 leader sequences of expression plasmid pCRU-K03 (Fig. 13; pCRU-K03 differs from pCRU-L01 only in the constant region of the light chain: in the pCRU-L01 the constant region is from a lambda-family, in the pCRU-K03 from a kappa-family, and the choice between these vectors can be made based on the family of the light chain that has been used in the screen, although it is also possible to clone V_L regions derived from a kappa family into a vector encoding a constant light chain of a lambda family and vice versa), or a plasmid derived from pCRU-K03, to obtain plasmids encoding a full length human IgG_1-kappa with binding specificities for Her-2. These plasmids are provisionally designated as pCRU-VK1HER2-1, pCRU-VK1HER2-2 and pCRU-VK1HER2-3, respectively.
Stable PER.C6™ derived cell lines are generated, according to methods known to the person skilled in the art, the cell lines expressing antibodies encoded by genetic information on either pCRU-VK1HER2-1, pCRU-VK1HER2-2 or pCRU-VK1HER2-3 and a

cell line expressing antibodies encoded by all three plasmids. Therefore, PER.C6™ cells are seeded in DMEM plus 10% FBS in tissue culture dishes (10 cm diameter) or T80 flasks with approximately 2.5×10^6 cells per dish and kept overnight under their normal culture conditions (10% CO_2 concentration and 37°C). The next day, transfections are performed in separate dishes at 37°C using Lipofectamine (Invitrogen Life Technologies) according to standard protocols provided by the manufacturer, with either 1-2 µg pCRU-VK1HER2-1, 1-2 µg pCRU-VK1HER2-2, 1-2 µg pCRU-VK1HER2-3 or 1 µg of a mixture of pCRU-VK1HER2-1, pCRU-VK1HER2-2 and pCRU-VK1HER2-3. As a control for transfection efficiency, a few dishes are transfected with a LacZ control vector, while a few dishes are not transfected and serve as negative controls.

After 5 hours waarom vorige vb 4 hrs en hier 5?, cells are washed twice with DMEM and refed with fresh medium without selection. The next day, medium is replaced with fresh medium containing 500 µg/ml G418. Cells are refreshed every 2 or 3 days with medium containing the same concentrations of G418. About 20-22 days after seeding, a large number of colonies are visible and from each transfection at least 300 are picked and grown via 96-well and/or 24-well via 6-well plates to T25 flasks. At this stage, cells are frozen (at least 1, but usually 4 vials per sub-cultured colony) and production levels of recombinant human IgG antibody are determined in the supernatant using an ELISA specific for human IgG_1. Also, at this stage G418 is removed from the culture medium and never re-applied again. For a representative number of colonies larger volumes are cultured to purify the recombinant human IgG_1 fraction from the conditioned supernatant using Protein A affinity chromatography according

to standard procedures. Purified human IgG_1 from the various clones is analyzed on SDS-PAGE, Iso-electric focusing (IEF), assayed binding to Her-2-Ig fusion proteins by ELISA, and analyzed for binding to Her-2 on the surface of BT474 cells by flow cytometry.

Clones obtained from the co-transfection of pCRU-VK1HER2-1, pCRU-VK1HER2-2 and pCRU-VK1HER2-3 are screened by PCR on genomic DNA for the presence or absence of each of the three constructs. The identity of the PCR products is further confirmed by DNA sequencing.

Next, it is demonstrated that a clonal cell line accounts for the production of each of the three binding specificities. Therefore, a limited number of colonies, which screened positive for the production of each of the three binding specificities (both by PCR at the DNA level as well as in the specified binding assays against Her-2, are subjected to single cell sorting using a fluorescence activated cell sorter (FACS) (Becton & Dickinson FACS VANTAGE SE). Alternatively, colonies are seeded at 0.3 cells/well to guarantee clonal outgrowth. Clonal cell populations, hereafter designated as sub-clones, are refreshed once a week with fresh medium. Sub-clones are grown and transferred from 96-wells via 24- and 6-wells plates to T25 flasks. At this stage, sub-clones are frozen (at least 1, but usually 4 vials per sub-clone) and production levels of recombinant human IgG_1 antibody are determined in the supernatant using a human IgG_1 specific ELISA. For a representative number of sub-clones, larger volumes are cultured to purify the recombinant human IgG_1 fraction from the conditioned supernatant using Protein A affinity chromatography according to standard procedures.

Purified human IgG_1 from the various sub-clones is subsequently analyzed as described above for human IgG_1 obtained from the parental clones, i.e. by SDS-PAGE, Iso-electric focusing (IEF) and binding to Her-2. Sub-clones will also be screened by PCR on genomic DNA for the presence or absence of each of the three constructs pCRU-VK1HER2-1, pCRU-VK1HER2-2 and pCRU-VK1HER2-3. The identity of the PCR products is further confirmed by DNA sequencing.

Other methods such as Southern blot and/or FISH can also be used to determine whether each of the three constructs are present in the clonal cell line.

Sub-clones that are proven to be transgenic for each of the three constructs are brought into culture for an extensive period to determine whether the presence of the transgenes is stable and whether expression of the antibody mixture remains the same, not only in terms of expression levels, but also for the ratio between the various antibodies that are secreted from the cell. Therefore, the sub-clone culture is maintained for at least 25 population doubling times either as an adherent culture or as a suspension culture. At every 4-6 population doublings, a specific production test is performed using the human IgG specific ELISA and larger volumes are cultured to obtain the cell pellet and the supernatant. The cell pellet is used to assess the presence of the three constructs in the genomic DNA, either via PCR, Southern blot and/or FISH. The supernatant is used to purify the recombinant human IgG_1 fraction as described supra. Purified human IgG_1 obtained at the various population doublings is analyzed as described, i.e. by SDS-PAGE, Iso-electric focusing (IEF) and binding to Her-2 by ELISA and by flow cytometry using BT474 cells.

Functionality of the antibody mixture of anti-Her-2 antibodies is analyzed in cell-based assays to determine whether the human IgG_1 mixture inhibits proliferation and/or induces apoptosis of BT474 cells. In addition the antibody mixtures are analyzed for their potential to induce antibody dependent cellular toxicity and complement dependent cytotoxicity of BT474 cells.

In each of the experiments described below the functionality of the antibody mixture recognizing Her-2 can be analyzed and compared to each of the individual IgG1 antibodies and to an equimolar combination of the three individual monospecific IgG1 molecules.

To assess the ability of the antibody mixtures to inhibit the proliferation of BT474 cells, these cells are allowed to adhere overnight in 96-well plates (1.5×10^5/well) and are subsequently incubated with several concentrations (5 - 20 μg/ml) of the antibody mixtures against Her-2 for 72 hours. The proliferation of the cells is measured by ^{3}H-thymidine incorporation during the last 6 hours of culture. Inhibition of growth is determined by plotting the percentage of ^{3}H-thymidine incorporation compared with untreated cells (taken as 100% reference value).

To analyze apoptosis induction of BT474 cells, these cells are allowed to adhere overnight in 48-well plates (2.5×10^5/well in 1ml) and are subsequently incubated with several concentrations (5 - 20 μg/ml) of the antibody mixtures against Her-2 for 4 hours. Hereafter the cells are harvested by trypsinization, are washed twice with PBS and are

incubated at RT for 10 min with 100 µl FITC-labelled annexin V (Caltag) diluted 1:25 in annexin V binding buffer (Caltag). Prior to the analysis of the samples by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA) propidium iodide (PI)(Sigma) is added to a final concentration of 5 µg/ml to distinguish necrotic cells (annexin V^{-}/PI^{+}) from apoptotic cells (annexin V^{+}/PI^{-}, early apoptotic cells; annexin V^{+}/PI^{+}, late apoptotic cells).

Antibody Dependent Cellular Cytotoxicity of the antibody mixtures is analyzed using peripheral blood mononuclear cells as effector cells and BT474 cells as target cells in a standard ^{51}Cr release assay as descibed supra (Huls et al, 1999). Complement dependent cytotoxicity is determined in a similar assay. Instead of the effector cells, now 50 µl human serum is added to the target cells. Subsequently, the assay is performed as described supra.
Alternatively, ADCC and CDC of the antibody mixtures is determined using a Europium release assay (Patel and Boyd, 1995) or using an LDH release assay (Shields et al, 2001).

The functionality of the antibody mixtures against Her-2 is also tested using in vivo animal models, such as for instance described in Spiridon et al, 2002.

Example 5

Expression of different functional human IgGs in the milk of transgenic animals

The V_H and V_H sequences of phages against proteins present on human B-cells, i.e. CD22 clone B28), CD72 (clone II-2) and HLA-DR (clone I-2) (Fig. 7) are cloned into expression plasmid pBC1 (as provided in the pBC1 Mouse Milk Expression System, Invitrogen Life Technologies) to obtain mammary-gland and lactation-specific expression of these human IgG molecules in transgenic animals, according to the manufacturers instructions. These mammary-gland specific expression vectors, encoding the antibody sequences for anti-CD22, anti-CD72 and anti-HLA-DR are introduced into the murine germline according to the manufacturers instructions. Such procedures have been described in detail previously (Platenburg et al., 1994). Obtained pups are screened for the presence of each of the three constructs by PCR on DNA isolated from the tail. Pups, either male or female, confirmed for being transgenic for each of the three antibodies, are weaned and matured. Female transgenic mice are fertilized at the age of 6-8 weeks and milk samples are obtained at several time points after gestation. Male transgenic mice are mated with non-transgenic females and female transgenic offspring (as determined with PCR as described above) is mated and milked as described above for the female transgenic founders. Whenever needed, female or male transgenic founders are mated for another generation to be able to obtain sufficient amounts of transgenic milk for each founder line. Transgenic milk is analyzed for the presence of human IgG with a human IgG specific ELISA, which does not cross-react with mouse IgG or other mouse milk components. Human IgG is purified from transgenic mouse milk

using Protein A affinity chromatography according to standard procedures. Purified human IgG is analyzed on SDS-PAGE, Isoelectric focusing and binding on the targets CD22, CD72 and HLA-DR. Functionality of the antibody mixture is analyzed as described supra.

Example 6 Production of an IgA/IgG mixture against a predefined target in a clonal PER.C6™ cell line

The V_H-V_L sequences of the phage UBS-54 directed against the homotypic adhesion molecule EP-CAM (Huls et al., 1999) was not only cloned into a vector encoding the constant domains of a human IgG1 with Kappa light chain (expression vector pUBS3000Neo), but also into an expression vector encoding the constant domains of a human IgA1 with Kappa light chain (expression vector pUBS54-IgA, Fig. 9). Hence, antibodies derived from pUBS3000Neo and pUBS54-IgA do bind to the same epitope on EPCAM. The only differences antibodies derived from pUBS3000Neo and pUBS54-IgA are in the sequences encoding the constant domains of the heavy chain, resulting in either an IgG_1 or IgA_1 isotype. The Kappa light chain sequences of these two vectors are identical.

Stable PER.C6™ derived cell lines expressing antibodies encoded by genetic information on pUBS3000Neo and pUBS54-IgA are generated by procedures well known to persons skilled in the art. Therefore, PER.C6™ cells are seeded in DMEM plus 10% FBS in tissue culture dishes (10 cm diameter) or T80 flasks with approximately 2.5 x 10^6 cells per dish and kept overnight under their normal culture conditions (10% CO_2 concentration and 37°C). The next day, transfections are performed in separate dishes at 37°C using Lipofectamine (Invitrogen Life Technologies) according to standard protocols provided by the manufacturer, with either 1-2 µg pUBS3000Neo and pUBS54-IgA. As a control for transfection efficiency, a few dishes are transfected with a LacZ control vector, while a few dishes are not transfected and serve as negative controls.

After 4-5 hours, cells are washed twice with DMEM and refed with fresh medium without selection. The next day, medium is

replaced with fresh medium containing 500 µg/ml G418. Cells are refreshed every 2 or 3 days with medium containing the same concentrations of G418. About 20-22 days after seeding, a large number of colonies is visible and from each transfection at least 300 are picked and grown via 96-well and/or 24-well via 6-well plates to T25 flasks. At this stage, cells are frozen (at least 1, but usually 4 vials per sub-cultured colony) and production levels of recombinant human IgG and human IgA antibody are determined in the supernatant using an ELISA specific for human IgG1 as well as an ELISA specific for human IgA. Also, at this stage G418 is removed from the culture medium and never re-applied again. For a representative number of colonies larger volumes are cultured to purify the recombinant human IgG_1 and human IgA fraction from the conditioned supernatant using for instance (a combination of) Protein L or LA affinity chromatography, cation exchange chromatography, hydrophobic interaction chromatography and gel filtration. Purified human immunoglobulins from the various clones are analyzed on SDS-PAGE, Iso-electric focusing (IEF) and binding to the target EPCAM using cell lines having a high expression of this molecule. The clones will also be screened by PCR on genomic DNA for the presence or absence of pUBS3000Neo and pUBS54-IgA. The identity of the PCR products is further confirmed by DNA sequencing.

A limited number of clones, which are screened positive for the production of both EPCAM IgG_1 and EPCAM IgA, are subjected to single cell sorting using a fluorescence activated cell sorter (FACS) (Becton Dickinson FACS VANTAGE SE). Alternatively, colonies are seeded at 0.3 cells/well to guarantee clonal outgrowth. Clonal cell populations, hereafter designated as sub-clones, are refreshed once a week

with fresh medium. Sub-clones are grown and transferred from 96-wells via 24- and 6-wells plates to T25 flasks. At this stage, sub-clones are frozen (at least 1, but usually 4 vials per sub-clone) and production levels of recombinant human IgG_1 and IgA antibody are determined in the supernatant using a human IgG_1 specific ELISA and a human IgA specific ELISA. For a representative number of sub-clones, larger volumes are cultured to purify the recombinant human IgG_1 and human IgA_1 fraction from the conditioned supernatant using for instance (a combination of) Protein L or LA affinity chromatography, cation exchange chromatography, hydrophobic interaction chromatography and gel filtration. Purified human immunoglobulins from the various clones are analyzed on SDS-PAGE, Iso-electric focusing (IEF) and binding to the target EPCAM using cell lines having a high expression of this molecule.

Sub-clones will also be screened by PCR on genomic DNA for the presence or absence of pUBS3000Neo and pUBS54-IgA. The identity of the PCR products is further confirmed by DNA sequencing.

Other methods such as Southern blot and/or FISH may also be used to determine whether both constructs are present in the clonal cell line.

Example 7 Production of a human IgG_1/IgG_3 mixture against multiple targets in a clonal PER.C6™ cell line

Phage clone UBS-54 and Clone K53 (Fig. 3) were obtained as described in Example 1. The V_H and V_L of clone UBS-54 was inserted into an expression vector containing the HAVT20 leader sequence and all the coding sequences for the constant domains of a human IgG_1 with a Kappa light chain by a method essentially as described (Boel et al, 2000). The resulting plasmid was designated as pUBS3000Neo (Fig. 4). It will be clear that expression vectors containing heavy chain constant domains of any desired isotype can be constructed by routine methods of molecular biology, using the sequences of these regions that are all available in the art. The V_H and V_L sequences of Phage clone K53 are cloned into an expression vector containing the HAVT20 leader sequence and all the coding sequences for the constant domains of a heavy chain of a human IgG_3 with a Kappa light chain by a method essentially as described (Boel et al, 2000). This expression vector is designated as pK53IgG3.

These plasmids are transiently expressed either alone or in combination in PER.C6™ cells. In brief, each 80 cm^2 flask is transfected by incubation for 4 hours with 140 µl lipofectamine + 10 µg DNA (either pUBS3000Neo, pK53IgG3 or 10 µg of both) in serum-free DMEM medium at 37°C. After 4 hours, this is replaced with DMEM + 10% FBS, and the cells are grown overnight at 37°C. Cells are then washed with PBS and the medium is replaced with Excell 525 medium (JRH Bioscience). The cells are allowed to grow at 37°C for 6 days, after which the cell culture supernatant is harvested. Human IgG specific ELISA analysis, i.e. measuring all IgG sub-types, is done to determine the IgG concentration in transfected and non-transfected PER.C6™ cells. Human IgG from each supernatant

is subsequently purified using Protein A affinity chromatography (Hightrap Protein A HP, cat.no. 1-040203) according to standard procedures, following recommendations of the manufacturer (Amersham Biosciences). After elution, samples are concentrated in a Microcon YM30 concentrator (Amicon) and buffer exchanged to 10 mM sodium phosphate, pH 6.7. Samples are analysed for binding to the targets EPCAM and CD46 using cell lines having a high expression of these molecules such as LS174T cells. Twelve µg of purified IgG, either transiently expressed UBS-54 IgG1, K53 IgG3 or IgG from the cells in which both antibodies were co-transfected, is subsequently analyzed on Isoelectric focusing gels (Serva Pre-cast IEF gels, pH range 3-10, cat.no. 42866). Samples are loaded on the low pH side and after focussing stained with colloidal blue. The pI values of the major isoforms for each sample are determined to illustrate whether there has been expression of UBS-54 IgG1, K53 IgG3 or bispecific heterodimers, depending on how the cells were transfected. The identification of heterodimers would indicate that single cells have translated both the IgG3 heavy chain of K53 and the IgG1 heavy chain of UBS-54 and assembled these into a full length IgG molecule together with the common light chain. The absence of bispecific heterodimers indicates that it is possible to translate both the IgG3 heavy chain of K53 and the IgG1 heavy chain of UBS-54 in single cells, but that these do not assemble into a full length IgG molecule together with the common light chain, i.e. there is preferential binding of IgG1 and IgG3 heavy chains. This could however also be explained by the lack of co-expression of UBS-54 IgG_1 and K53 IgG_3. Therefore, stable clonal cell lines expressing both pUBS3000Neo, pK53IgG3 are generated by procedures well known to persons skilled in the art. PER.C6™

cells are seeded in DMEM plus 10% FBS in tissue culture dishes (10 cm diameter) or T80 flasks with approximately 2.5 x 10^6 cells per dish and kept overnight under their normal culture conditions (10% CO_2 concentration and 37°C). The next day, transfections are performed in separate dishes at 37°C using Lipofectamine (Invitrogen Life Technologies) according to standard protocols provided by the manufacturer, with either 1-2 µg pUBS3000Neo, pK53IgG3 or both. As a control for transfection efficiency, a few dishes are transfected with a LacZ control vector, while a few dishes will be not transfected and serve as negative controls.

After 4-5 hours, cells are washed twice with DMEM and refed with fresh medium without selection. The next day, medium is replaced with fresh medium containing 500 µg/ml G418. Cells are refreshed every 2 or 3 days with medium containing the same concentrations of G418. About 20-22 days after seeding, a large number of colonies is visible and from each transfection at least 300 are picked and grown via 96-well and/or 24-well via 6-well plates to T25 flasks. At this stage, cells are frozen (at least 1, but usually 4 vials per sub-cultured colony) and production levels of recombinant human IgG antibody are determined in the supernatant using an ELISA specific for all sub-types of human IgG. Also, at this stage G418 is removed from the culture medium and never re-applied again. For a representative number of colonies larger volumes are cultured to purify the recombinant human IgG from the conditioned supernatant using Protein A affinity chromatography (Hightrap Protein A HP, cat.no. 1-040203) according to standard procedures, following recommendations of the manufacturer (Amersham Biosciences). Purified human immunoglobulins from the various clones are analyzed on SDS-PAGE, Iso-electric focusing (IEF) and binding to the targets

EPCAM and CD46 using cell lines having a high expression of these molecules such as LS174T cells. The clones are also screened by PCR on genomic DNA for the presence or absence of pUBS3000Neo and pK53IgG3. The identity of the PCR products is further confirmed by DNA sequencing.

A limited number of clones, which are screened positive for the production of both EPCAM IgG1 and K53 IgG3, are subjected to single cell sorting using a fluorescence activated cell sorter (FACS) (Becton Dickinson FACS VANTAGE SE).

Alternatively, colonies are seeded at 0.3 cells/well to guarantee clonal outgrowth. Clonal cell populations, hereafter designated as sub-clones, are refreshed once a week with fresh medium. Sub-clones are grown and transferred from 96-wells via 24- and 6-wells plates to T25 flasks. At this stage, sub-clones are frozen (at least 1, but usually 4 vials per sub-clone) and production levels of recombinant human IgG antibody are determined in the supernatant using a human IgG specific ELISA. For a representative number of sub-clones, larger volumes are cultured to purify the recombinant human IgG fraction from the conditioned supernatant using Protein A affinity chromatography (Hightrap Protein A HP, cat.no. 1-040203) according to standard procedures, following recommendations of the manufacturer (Amersham Biosciences). Purified human immunoglobulins from the various clones are analyzed on SDS-PAGE, Iso-electric focusing (IEF) and binding to the targets EPCAM and CD46 using cell lines having a high expression of this molecules, such as for instance LS174T cells, or transfectants expressing these molecules.

Sub-clones are also screened by PCR on genomic DNA for the presence or absence of pUBS3000Neo and pK53IgG3. The identity of the PCR products is further confirmed by DNA sequencing. Other methods such as Southern blot and/or FISH may also be

used to determine whether both constructs are present in the clonal cell line.

Once the clonal sub-clones are available and confirmed positive for the expression of both UBS-54 IgG1 and K53 IgG3, the presence of functional K53 and UBS-54 shows that it is possible to generate a mixture of functional IgG's with different isotypes with the common light chain in a single cell. Analysis of the expression of bispecific antibodies binding both EpCAM and CD46 will reveal to what extent the different heavy chains having a different subtype will pair, which will influence the amount of bispecific antibodies produced.

Example 8. Selection of phage carrying single chain Fv fragments specifically recognizing rabies virus glyco protein (RVGP) using RVGP-Ig fusion protein, and expression of mixtures of antibodies against the rabies virus.

This example describes the production of mixtures of antibodies against the rabies virus, as another potential target. As an antigen, the Rabies Virus Glycoprotein (RVGP) is chosen, but other rabies antigens may be chosen or included as well for this purpose. Several monoclonal antibodies recognizing RVGP have already been described in the art, and polyclonal antibodies have been recognized to be useful in treatment of rabies infections as well (see e.g. Schumacher et al, 1989; WO 89/09789; EP0402029; EP0445625; US patent 5695757).

Antibody fragments are selected using antibody phage display libraries and MAbstract™ technology, essentially as described in US patent 6,265,150 and in WO 98/15833. All procedures are performed at room temperature unless stated otherwise. The sequence of RVGP is available to the person skilled in the art, for cloning purposes, see e.g. Anilionis et al, 1982; Yelverton et al, 1983; Kieny et al, 1984. An RVGP-Ig fusion protein consisting of whole RVGP fused genetically to the CH2 and CH3 domains of human IgG1 is produced using vector pcDNA3.1 Zeo-CH2-CH3 expressed in PER.C6™ and coated for 2 hours at 37° onto the surface of Maxisorp™ plastic tubes (Nunc) at a concentration of 1.25 µg/ml. The tubes are blocked for 1 h in 2% fat free milk powder dissolved in PBS (MPBS). Simultaneously, 500 µl (approximately 10^{13} cfu) of a phage display library expressing single chain Fv fragments (scFv's) essentially prepared as described by De Kruif et al (1995a,b) and references therein, is added to two volumes of 4% MPBS. In

this experiment, selections are performed using fractions of the original library constructed using only one single variable light chain gene species (e.g. a 'VK1'-library). In addition, human serum is added to a final concentration of 15% and blocking is allowed to proceed for 30-60 min. The RVGP-Ig-coated tubes are emptied and the blocked phage library is added. The tube is sealed and rotated slowly for 1 h, followed by 2 h of incubation without rotation. The tubes are emptied and washed 10 times in PBS containing 0.1% Tween-20, followed by washing 5 times in PBS. 1 ml glycine-HCL, 0.05 M, pH 2.2 is added, and the tube is rotated slowly for 10 min. The eluted phages are added to 500 µl 1M Tris-HCl pH 7.4. To this mixture, 3.5 ml of exponentially growing XL-1 blue bacterial culture is added. The tubes are incubated for 30 min at 37°C without shaking. Then, the bacteria are plated on 2TY agar plates containing ampicillin, tetracycline and glucose. After overnight incubation of the plates at 37°C, the colonies are scraped from the plates and used to prepare an enriched phage library, essentially as described by De Kruif et al. (1995a,b). Briefly, scraped bacteria are used to inoculate 2TY medium containing ampicillin, tetracycline and glucose and grown at a temperature of 37°C to an OD_{600nm} of ~0.3. Helper phages are added and allowed to infect the bacteria after which the medium is changed to 2TY containing ampicillin, tetracycline and kanamycin. Incubation is continued overnight at 30°C. The next day, the bacteria are removed from the 2TY medium by centrifugation after which the phages are precipitated using polyethylene glycol 6000/NaCl. Finally, the phages are dissolved in a small volume of PBS-1% BSA, filter-sterilized and used for a next round of selection. The selection/re-infection procedure is performed twice. After the second round of selection, individual *E.coli*

colonies are used to prepare monoclonal phage antibodies. Essentially, individual colonies are grown to log-phase and infected with helper phages after which phage antibody production is allowed to proceed overnight. Phage antibody containing supernatants are tested in ELISA for binding activity to human RVGP-Ig coated 96 wells plates.

Selected phage antibodies that are obtained in the screen described above, are validated in ELISA for specificity. For this purpose, human RVGP-Ig is coated to Maxisorp ELISA plates. After coating, the plates are blocked in 2% MPBS. The selected phage antibodies are incubated in an equal volume of 4% MPBS. The plates are emptied, washed once in PBS, after which the blocked phages are added. Incubation is allowed to proceed for 1 h, the plates are washed in PBS 0.1% Tween-20 and bound phages are detected using an anti-M13 antibody conjugated to peroxidase. As a control, the procedure is performed simultaneously using a control phage antibody directed against thyroglobulin (De Kruif et al. 1995a,b), which serves as a negative control.

The phage antibodies that bind to human RVGP-Ig are subsequently tested for binding to human serum IgG to exclude the possibility that they recognized the Fc part of the fusion protein.

In another assay the phage antibodies are analyzed for their ability to bind PER.C6™ cells that express RVGP To this purpose PER.C6™ cells are transfected with a plasmid carrying a cDNA sequence encoding RVGP or with the empty vector and stable transfectants are selected using standard techniques known to a person skilled in the art (e.g. Coligan, J.E. etal. (2001) Current protocols in protein science, volume I. John Wiley & Sons, Inc. New York). For

flow cytometry analysis, phage antibodies are first blocked in an equal volume of 4% MPBS for 15 min at 4°C prior to the staining of the RVGP- and control transfected PER.C6™ cells. The blocked phages are added to a mixture of unlabelled control transfected PER.C6™ cells and RGVP transfected PER.C6™ cells that have been labelled green using a lipophylic dye (PKH67, Sigma). The binding of the phage antibodies to the cells is visualized using a biotinylated anti-M13 antibody (Santa Cruz Biotechnology) followed by streptavidin-phycoerythrin (Caltag). Anti RVGP scFv selectively stains the PER.C6™ RVGP transfectant while they do not bind the control transfectant.

An alternative way of screening for phages carrying single chain Fv fragments specifically recognizing human RVGP, is by use of RVGP-transfected PER.C6™ cells. PER.C6™ cells expressing membrane bound RVGP are produced as described supra. Phage selection experiments are performed as described supra, using these cells as target. A fraction of the phage library comprised of scFv-phage particles using only one single scFv species (500 µl, approximately 10^{13} cfu) is blocked with 2 ml RPMI/10%FCS/1%NHS for 15′ at RT. Untransfected PER.C6™ cells (~$10*10^{6}$ cells) are added to the PER.C6-RVGP cells (~$1.0*10^{6}$ cells). This mixture is added to the blocked light chain restricted phage-library and incubated for 2,5 hr while slowly rotating at 4°C. Subsequently, the cells are washed twice and were resuspended in 500µl RPMI/10%FCS and incubated with a murine anti-RVGP antibody (Becton Dickinson) followed by a phycoerythrin (PE)-conjugated anti-mouse-IgG antibody (Calltag) for 15′ on ice. The cells are washed once and transferred to a 4 ml tube. Cell sorting is performed on a FACSvantage fluorescence-

activated cell sorter (Becton Dickinson) and RVGP (PE positive) cells are sorted. The sorted cells are spun down, the supernatant is saved and the bound phages are eluted from the cells by resuspending the cells in 500µl 50mM Glycin pH2.2 followed by incubation for 5 min. at room temperature. The mixture is neutralized with 250µl 1M Tris-HCl pH 7.4 and added to the rescued supernatant. Collectively these phages are used to prepare an enriched phage library as described above. The selection/re-infection procedure is performed twice. After the second round of selection, monoclonal phage antibodies are prepared and tested for binding to RVGP-PER.C6™ cells and untransfected PER.C6™ cells as described supra. Phages that are positive on RVGP-transfected cells are subsequently tested for binding to the RVGP-IgG fusion protein in ELISA as described supra.

The selected scFv fragments are cloned in a human IgG1 format, according to methods known in the art (e.g. Boel et al, 2000). To this purpose, the VL fragment shared by the selected scFv is PCR amplified using oligo's that add appropriate restriction sites. A similar procedure is used for the VH genes. Thus modified genes are cloned in expression vector pCRU-L01 or pCRU-K03, or derivatives thereof, which results in expression vectors encoding a complete huIgG1 heavy chain and a complete human light chain gene having the same specificity as the original phage clone. By this method, three different heavy chains are cloned into separate expression vectors, while only one of the vectors needs to comprise the common light chain sequence. These expression vectors are provisionally designated pCRU-RVGP-1, pCU-RVGP-2, and pCRU-RVGP-3. Alternatively, these three vectors may lack DNA encoding the V_L region, which can then

be encoded in a fourth, separate expression vector not encoding a heavy chain. It is also possible to have V_L sequences present in all three or two of the three vectors comprising the different V_H sequences.

Stable PER.C6™ derived cell lines are generated, according to methods known to the person skilled in the art (see e.g. WO 00/63403), the cell lines expressing antibodies encoded by genetic information on either pCRU-RVGP-1, pCRU-RVGP-2 or pCRU-RVGP-3 and a cell line expressing antibodies encoded by all three plasmids. Therefore, PER.C6™ cells are seeded in DMEM plus 10% FBS in tissue culture dishes (10 cm diameter) or T80 flasks with approximately 2.5 x 10^6 cells per dish and kept overnight under their normal culture conditions (10% CO_2 concentration and 37°C). The next day, transfections are performed in separate dishes at 37°C using Lipofectamine (Invitrogen Life Technologies) according to standard protocols provided by the manufacturer, with either 1-2 µg pCRU-RVGP-1, 1-2 µg pCRU-RVGP-2, 1-2 µg pCRU-RVGP-3 or 1 µg of a mixture of pCRU-RVGP-1, pCRU-RVGP-2 and pCRU-RVGP-3. As a control for transfection efficiency, a few dishes are transfected with a LacZ control vector, while a few dishes will be not transfected and serve as negative controls. After 4 to 5 hours, cells are washed twice with DMEM and refed with fresh medium without selection. The next day, medium are replaced with fresh medium containing 500 µg/ml G418. Cells are refreshed every 2 or 3 days with medium containing the same concentrations of G418. About 20-22 days after seeding, a large number of colonies are visible and from each transfection at least 300 are picked and grown via 96-well and/or 24-well via 6-well plates to T25 flasks. At this stage, cells are frozen (at least 1, but usually 4 vials per sub-cultured colony) and production levels of recombinant

human IgG antibody are determined in the supernatant using an ELISA specific for human IgG_1 (described in WO 00/63403). Also, at this stage G418 is removed from the culture medium and never re-applied again. For a representative number of colonies larger volumes will be cultured to purify the recombinant human IgG_1 fraction from the conditioned supernatant using Protein A affinity chromatography according to standard procedures. Purified human IgG_1 from the various clones is analyzed on SDS-PAGE, Iso-electric focusing (IEF) and binding to the target RVGP using an RVGP PER.C6-transfectant described above.

Colonies obtained from the co-transfection with pCRU-RVGP-1, pCRU-RVGP-2 and pCRU-RVGP-3 are screened by PCR on genomic DNA for the presence or absence of each of the three constructs. The identity of the PCR products is further confirmed by DNA sequencing.

A limited number of colonies, which screened positive for the production of each of the three binding specificities (both by PCR at the DNA level as well as in the specified binding assays against RVGP), are subjected to single cell sorting using a fluorescence activated cell sorter (FACS) (Becton & Dickinson FACS VANTAGE SE). Alternatively, colonies are seeded at 0.3 cells/well to guarantee clonal outgrowth. Clonal cell populations, hereafter designated as sub-clones, are refreshed once a week with fresh medium. Sub-clones are grown and transferred from 96-wells via 24- and 6-wells plates to T25 flasks. At this stage, sub-clones are frozen (at least 1, but usually 4 vials per sub-clone) and production levels of recombinant human IgG_1 antibody are determined in the supernatant using a human IgG_1 specific ELISA. For a representative number of sub-clones, larger volumes are cultured to purify the recombinant human IgG_1

fraction from the conditioned supernatant using Protein A affinity chromatography according to standard procedures. Purified human IgG_1 from the various sub-clones is subsequently analyzed as described above for human IgG_1 obtained from the parental clones, i.e. by SDS-PAGE, Iso-electric focusing (IEF) and binding to the target RVGP. Sub-clones are also screened by PCR on genomic DNA for the presence or absence of each of the three constructs pCRU-RVGP-1, pCRU-RVGP-2 and pCRU-RVGP-3. The identity of the PCR products is further confirmed by DNA sequencing.

Other methods such as Southern blot and/or FISH can also be used to determine whether each of the three constructs are present in the clonal cell line.

Sub-clones that are proven to be transgenic for each of the three constructs are brought into culture for an extensive period to determine whether the presence of the transgenes is stable and whether expression of the antibody mixture remains the same, not only in terms of expression levels, but also for the ratio between the various antibody isoforms that are secreted from the cell. Therefore, the sub-clone culture is maintained for at least 25 population doubling times either as an adherent culture or as a suspension culture. At every 4-6 population doublings, a specific production test is performed using the human IgG specific ELISA and larger volumes are cultured to obtain the cell pellet and the supernatant. The cell pellet is used to assess the presence of the three constructs in the genomic DNA, either via PCR, Southern blot and/or FISH. The supernatant is used to purify the recombinant human IgG_1 fraction as described supra.

Purified human IgG_1 obtained at the various population doublings is analyzed as described, i.e. by SDS-PAGE, Iso-electric focusing (IEF) and binding to the target RVGP.

The efficacy of the antibody mixtures against rabies is tested in in vitro cell culture assays where the decrease in spread of rabies virus is measured, as well as in in vivo animal models infected by rabies. Such models are known to the person skilled in the art, and are e.g. described in Schumacher et al, 1989; WO 89/09789; EP0402029; EP0445625; US patent 5695757.

References

Appel RD, Bairoch A, Hochstrasser DF. 1994. A new generation of information retrieval tools for biologists: the example of the ExPASy WWW server. Trends Biochem. Sci. 19: 258-260.

Amstutz P, Forrer P, Zahnd C, Plückthun A. 2001. In vitro display technologies: Novel developments and applications. Curr. Opin. Biotechnol. 12: 400-405.

Anilionis A, Wunner WH, Curtis PJ. 1982. Amino acid sequence of the rabies virus glycoprotein deduced from its cloned gene. Comp Immunol Microbiol Infect Dis 5: 27-32.

Archer JS, Kennan WS, Gould MN, Bremel RD. 1994. Human growth hormone (hGH) secretion in milk of goats after direct transfer of the hGH gene into the mammary gland by using replication-defective retrovirus vectors. Proc Natl Acad Sci U S A. 91: 6840-6844.

Baecher-Allen CM, Santora K, Sarantopoulos S, Den W, Sompuram SR, Cevallos AM, Bhat N, Ward H, Sharon J. 1999. Generation of a polyclonal Fab phage display library to the protozoan parasite Cryptosporidium parvum. Comb. Chem. High Throughput Screen. 2: 319-325.

Bendig MM. 1988. The production of foreign proteins in mammalian cells. Genet Eng 7: 91-127.

Boel E, Verlaan S, Poppelier MJ, Westerdaal NA, Van Strijp JA, Logtenberg T. 2000. Functional human monoclonal antibodies of all isotypes constructed from phage display library-derived single-chain Fv antibody fragments. J Immunol

Methods 239:153-166.

Brink MF, Bishop MD, Pieper FR. 2000. Developing efficient strategies for the generation of transgenic cattle which produce biopharmaceuticals in milk. Theriogenology 53: 139-148.

Burton DR and Barbas CF (1994) Human antibodies from combinatorial libraries. Adv Immunol 57:191-280

Campbell KH, McWhir J, Ritchie WA, Wilmut I. 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature 380: 64-66.

Casellas R, Shih TA, Kleinewietfelt M, Rakoniac J, Nemazee D, Rajewski K, Nussenzweig MC. 2001. Contribution of receptor editing to the antibody repertoire. Science 291: 1541-1544.

Chester KA, Begent RH, Robson L, Keep P, Pedley RB, Boden JA, Green A, Winter G, Cochet O, et al. 1994. Phage libraries for generation of clinically useful antibodies. Lancet 343: 455-456.

Cockett MI, Bebbington CR, Yarranton GT. 1990. High level expression of tissue inhibitor of metalloproteinases in Chinese hamster ovary cells using glutamate synthetase gene amplification. Bio/technology 8: 662-667.

Den W, Sompuram SR, Sarantopoulos S, Sharon J. 1999. A bi-directional phage display vector for the selection and mass transfer of polyclonal antibody libraries. J. Immunol. Meth. 222: 45-57.

De Kruif J, Terstappen L, Boel E and Logtenberg T (1995a) Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc Natl Acad Sci USA 92:3938

De Kruif J, Boel E and Logtenberg T (1995b) Selection and application of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. J Mol Biol 248:97

De Kruif J, Van der Vuurst de Vries AR, Cilenti L, Boel E, Van Ewijk W and Logtenberg T (1996) New perspectives on recombinant human antibodies. Immunol Today 17:453

Dinnyes A, De Sousa P, King T, Wilmut I. 2002. Somatic cell nuclear transfer: recent progress and challenges. Cloning Stem Cells 4: 81-90.

Fanger MW, Segal DM, Romet-Lemonne JL. 1991. Bispecific antibodies and targeted cellular cytotoxicity. Immunol Today. 12:51-54.

Flavell DJ, Noss A, Pulford KA, Ling N, Flavell SU. 1997. Systemic therapy with 3BIT, a triple combination cocktail of anti-CD19, -CD22, and -CD38-saporin immunotoxins, is curative of human B-cell lymphoma in severe combined immunodeficient mice. Cancer Res 57: 4824-4829.

Fischer R, Hoffmann K, Schillberg S, Emans N. 2000. Antibody production by molecular pharming in plants. J Biol Regul Homeost Agents 14: 83-92.

Fishwild DM, O'Donnell SL, Bengoechea T, Hudson DV, Harding F, Bernhard SL, Jones D, Kay RM, Higgins KM, Schramm SR, Lonberg N. 1996. High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. Nat Biotechnol.14: 845-51.

Ghetie M-A, Podar EM, Ilgen A, Gordon BE, Uhr JW, Vitetta, ES. 1997. Homodimerization of tumor-reactive monoclonal antibodies markedly increases their ability to induce growth arrest or apoptosis of tumor cells. Proc. Natl. Acad. Sci. USA 94: 7509-7514.

Giddings G, Allison G, Brooks D, Carter A. 2000. Transgenic plants as factories for biopharmaceuticals. Nat. Biotechnol. 18: 1151-1155.

Gorman C, Bullock C. 2000. Site-specific gene targeting for gene expression in eukaryotes. Curr Opin Biotechnol 11: 455-460.

Harrison JL, Williams SC, Winter G, Nissim A. 1996. Screening of phage antibody libraries. Methods Enzymol. 267: 83-109.

Herpunjaa A, Junnikkala S, Meris S. 2000. Rituximab (anti-CD20) therapy of B-cell lymphomas: direct complement killing is superior to cellular effector mechanisms. Scand. J. Immunol. 51: 634-641.

Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature. 342 : 76-78.

Hoogenboom HR, Winter G. 1992. By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. J. Mol. Biol. 227: 381-388.

Huls, G. A., Heijnen, I. A., Cuomo, M. E., Koningsberger, J. C., Wiegman, L., Boel, E., van der Vuurst de Vries, A. R., Loyson, S. A., Helfrich, W., van Berge Henegouwen, G. P., van Meijer, M., de Kruif, J. & Logtenberg, T. 1999. A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. Nat Biotechnol 17: 276-281.

Ishida I, Tomikuza K, Yoshida H, Tahara T, Takahashi N, Ohguma A, Tanaka S, Umehashi M, Maeda H, Nozaki C, Halk E, Lonberg N. 2002. Production of human monoclonal and polyclonal antibodies in TransChromo animals. Cloning and Stem Cells 4: 91-102.

Jespers LS, Roberts A, Mahler SM, Winter G, Hoogenboom HR. 1994. Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen. Biotechnology (N Y) 12: 899-903.

Kieny MP, Lathe R, Drillien R, Spehner D, Skory S, Schmitt D, Wiktor T, Koprowski H, Lecocq JP. 1984. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 312: 163-166.

Kim SJ, Kim Ns, Ryu CJ, Hong HJ, Lee GM. 1998. Characterization of chimeric antibody producing CHO cells in the course of dihydrofolate reductase-mediated gene

amplification and their stability in the absence of selective pressure. Biotechnol Bioeng 58: 73-84.

Kinoshita T, Nagai H, Murate T, Saito H. 1998. CD20-negative relapse in B-cell lymphoma after treatment with Rituximab. J. Clin. Oncol. 16: 3916.

Kohler G and Millstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497.

Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84: 1415-1420.

Kroesen BJ, ter Haar A, Spakman H, Willemse P, Sleijfer DT, de Vries EG, Mulder NH, Berendsen HH, Limburg PC, The TH, et al. 1993. Local antitumour treatment in carcinoma patients with bispecific-monoclonal-antibody-redirected T cells. Cancer Immunol Immunother.37: 400-407.

Kroesen BJ, Buter J, Sleijfer DT, Janssen RA, van der Graaf WT, The TH, de Leij L, Mulder NH. 1994. Phase I study of intravenously applied bispecific antibody in renal cell cancer patients receiving subcutaneous interleukin 2. Br J Cancer. 70:652-661.

Larrick JW, Thomas DW. 2001. Producing proteins in transgenic plants and animals. Curr. Opin. Biotechnol. 12: 411-418.

Larrick JW, Yu L, Naftzger C, Jaiswal S, Wycoff K. 2001. Production of secretory IgA antibodies in plants. Biomol. Eng. 18: 87-94.

Le Doussal JM, Barbet J, Delaage M. 1992. Bispecific-antibody-mediated targeting of radiolabeled bivalent haptens: theoretical, experimental and clinical results. Int J Cancer Suppl. 7:58-62.

Limonta J, Pedraza A, Rodriguez A, Freyre FM, Barral AM, Castro FO, Lleonart R, Gracia CA, Gavilondo JV, de la Fuente J. 1995. Production of active anti-CD6 mouse/human chimeric antibodies in the milk of transgenic mice. Immunotechnology. 1: 107-113.

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G. 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on a phage. J. Mol. Biol. 222: 581-597.

Marks JD, Ouwehand WH, Bye JM, Finnern R, Gorick BD, Voak D, Thorpe SJ, Hughes-Jones NC, Winter G. 1993. Human antibody fragments specific for human blood group antigens from a phage display library. Biotechnology (NY) 11: 1145-1149.

Massengale WT, McBurney E, Gurtler J. 2002. CD20-negative relapse of cutaneous B-cell lymphoma after anti-CD20 monoclonal antibody therapy. J. Am. Acad. Dermatol. 46: 441-443.

McCafferty J, Griffiths AD, Winter G, Chiswell DJ. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348: 552-554.

Mendez MJ, Green LL, Corvalan JR, Jia XC, Maynard-Currie CE, Yang XD, Gallo ML, Louie DM, Lee DV, Erickson KL, Luna J, Roy CM, Abderrahim H, Kirschenbaum F, Noguchi M, Smith DH, Fukushima A, Hales JF, Klapholz S, Finer MH, Davis CG, Zsebo KM, Jakobovits A. 1997. Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice.
Nat Genet. 15: 146-56.

Merchant AM, Zhu Z, Yuan JQ, Goddard A, Adams CW, Presta LG, Carter P. 1998. An efficient route to human bispecific IgG. Nat. Biotech. 16: 677-681.

Mössner E, Plückthun A. 2001. Directed evolution with fast and efficient selection technologies. Chimia 55: 325-329.

Nemazee D. 2000. Receptor editing in B cells. Adv. Immunol. 74: 89-126.

Nemazee D, Weigert M. 2000. Revising B cell receptors. J. Exp. Med. 191: 1813-1817.

Nissim A, Hoogenboom HR, Tomlinson IM, Flynn G, Midgley C, Lane D, Winter G. 1994. Antibody fragments from a "single pot" phage display library as immunological reagents. EMBO J. 13: 692-698.

Patel AK, Boyd PN. 1995. An improved assay for antibody dependent cellular cytotoxicity based on time resolved fluorometry. Journal of Immunological Methods 184: 29-38.

Peeters K, De Wilde C, De Jaeger G, Angenon G, Depicker A. 2001. Production of antibodies and antibody fragments in plants. Vaccine 19: 2756-2761.

Platenburg GJ, Kootwijk EP, Kooiman PM, Woloshuk SL, Nuijens JH, Krimpenfort PJ, Pieper FR, de Boer HA, Strijker R. 1994. Expression of human lactoferrin in milk of transgenic mice. Transgenic Res 3: 99-108.

Pollock DP, Kutzko JP, Birck-Wilson E, Williams JL, Echelard Y, Meade HM. 1999. Transgenic milk as a method for the production of recombinant antibodies. J Immunol Methods. 231: 147-157.

Radic MC, Mascelli MA, Shan H, Weigert M. 1991. Ig H and L chain contributions to autoimmune specificities. J. Immunol. 146: 176-182.

Santorana KE, Sarantopoulos S, Den W, Petersen-Mahrt S, Sompuram SR, Sharon J. 2000. Generation of a polyclonal Fab phage display library to the human breast carcinoma cell line BT-20. Comb. Chem. High Throughput Screen. 3: 51-57.

Schaffitzel C, Hanes J, Jermutus L, Pluckthun A. 1999. Ribosome display: an in vitro method for selection and evolution of antibodies from libraries. J Immunol Methods 231: 119-135.

Schmitz K, Brugger W, Weiss B, Kaiserling E, Kanz L. 1999. Clonal selection of CD20-negative non-Hodgkin's lymphoma cells after treatment with anti-CD20 antibody rituximab. Br. J. Haematol. 106: 571-572.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science. 278: 2130-2133.

Schumacher CL, Dietzschold B, Ertl HC, Niu HS, Rupprecht CE, Koprowski H. 1989. Use of mouse anti-rabies monoclonal antibodies in postexposure treatment of rabies. J Clin Invest. 84: 971-975.

Segal DM, Weiner GJ, Weiner LM. 1999. Bispecific antibodies in cancer therapy. Curr Opin Immunol. 11:558-562.

Segal DM, Weiner GJ, Weiner LM. 2001. Introduction: bispecific antibodies. J Immunol Methods. 248:1-6.

Shields, RL, Namenuk AK, Hong K, Gloria Meng Y, Rae J, Biggs J, Xie D, Lai J, Stadlen A, Li B, Fox JA and Presta LG. 2001. High resolution mapping of the binding site on human IgG1 for FcgRI, FcgRII, FcgRIII and FcRn and design of IgG1 variants with improved binding to the FcgR. J Biol Chem 276: 6591-6604.

Spiridon CI, Ghetie MA, Uhr J, Marches R, Li JL, Shen GL, Vitetta ES. 2002. Tartgeting multiple her-2 epitopes with monoclonal antibodies results in improved antigrowth activity

of a human breast cancer cell line in vitro and in vivo. Clin. Cancer Res. 8: 1720-1730.

Van der Vuurst de Vries A, Logtenberg T. 1999. Dissecting the human peripheral B-cell compartment with phage display-derived antibodies. Immunology 98: 55-62.

van Kuik-Romeijn P, de Groot N, Hooijberg E, de Boer HA. 2000. Expression of a functional mouse-human chimeric anti-CD19 antibody in the milk of transgenic mice. Transgenic Res. 9: 155-159.

Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, McCafferty J, Hodits RA, Wilton J, Johnson KS. 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat. Biotech. 14: 309-314.

Weiner GJ, De Gast GC. 1995. Bispecific monoclonal antibody therapy of B-cell malignancy. Leuk Lymphoma. 16:199-207.

Weiner LM. 2000. Bispecific antibodies in cancer therapy. Cancer J Sci Am. Suppl 3:S265-271.

Wilmut I. Clark AJ. 1991. Basic techniques for transgenesis. J Reprod Fertil Suppl 43: 265-275.

Wilmut I, Archibald AL, McClenaghan M, Simons JP, Whitelaw CB, Clark AJ. 1991. Experentia 47: 905-912.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810-813.

Wilson TJ, Kola I. 2001. The LoxP/CRE system and genome modification. Methods Mol Biol. 158:83-94.

Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. 1994. Making antibodies by phage display technology. Annu. Rev. Immunol. 12: 433-455.

Yelverton E, Norton S, Obijeski JF, Goeddel DV. 1983. Rabies virus glycoprotein analogs: biosynthesis in Escherichia coli. Science 219: 614-620.

Yoo EM, Coloma MJ, Trinh KR, Nguyen TQ, Vuong LU, Morrison SL, Chintalacharuvu KR. 1999. Structural requirements for polymeric immunoglobulin assembly and association with J chain. J Biol Chem. 274: 33771-33777.

Claims

1. A method for production of a mixture of antibodies in a recombinant host, the method comprising the step of: expressing in a recombinant host cell a nucleic acid sequence or nucleic acid sequences encoding at least one light chain and at least three different heavy chains that are capable of pairing with said at least one light chain.

2. A method according to claim 1, wherein said recombinant host cell comprises a nucleic acid sequence encoding a common light chain that is capable of pairing with said at least three different heavy chains, such that the produced antibodies comprise common light chains.

3. A method according to claim 1 or 2, further comprising the step of:
recovering the antibodies from the host cell or the host cell culture.

4. A method according to any one of claims 1-3, wherein at least two antibodies comprising a heavy-light chain dimer in said mixture of antibodies have different specificities and/or affinities.

5. A method according to any one of claims 1-4, wherein said recombinant host cell is capable of high-level expression of recombinant proteins without the necessity of amplification of the nucleic acid encoding said proteins in said cell.

6. A method according to any one of claims 1-5, wherein said cell is derived from a human embryonic retina cell that has been immortalized or transformed by adenoviral E1 sequences.

7. A method according to claim 6, wherein said host cell is derived from a PER.C6 cell.

8. A mixture of antibodies that is obtainable by a method according to any one of claims 1-7.

9. A mixture according to claim 8, wherein antibodies present in said mixture bind to different epitopes of the same antigen and/or to different antigens present in one antigen comprising mixture.

10. A recombinant host cell comprising a nucleic acid sequence encoding a light chain and nucleic acid sequences encoding at least three different heavy chains of an antibody, wherein said light and heavy chains are capable of pairing.

11. A composition comprising a mixture of recombinantly produced antibodies, wherein at least three different heavy chain sequences are represented in the mixture of recombinant antibodies.

12. A composition according to claim 11, wherein the light chains of said antibodies comprise a common sequence.

13. A composition according to claim 11 or 12, wherein said mixture comprises bispecific antibodies.

14. A composition according to any one of claims 11-13, wherein said mixture of antibodies has been produced by recombinant host cells according to claim 10.

15. A composition according to any one of claims 11-14, wherein at least two of said antibodies have different specificities.

16. A composition according to claim 15, wherein said different specificities are directed to different epitopes on the same antigen.

17. A composition according to claim 15, wherein said different specificities are directed to different antigens present in one antigen comprising mixture.

18. A composition according to any one of claims 11-14, wherein at least two of said antibodies have different affinities for the same epitope.

19. A composition according to any one of claims 11-18, wherein said composition has an effect that is greater than the effect of each individual antibody present in said composition, wherein said effect is measured in a functional assay.

20. A method for identifying at least one host cell clone that produces a mixture of antibodies, wherein said mixture of antibodies has a desired effect according to a

functional assay, the method comprising the steps of:
(i) providing a host cell with nucleic acid sequence encoding at least one light chain and nucleic acid sequences encoding at least two different heavy chains, wherein said heavy and light chains are capable of pairing with each other;
(ii) culturing at least one clone of said host cell under conditions conducive to expression of said nucleic acid sequences;
(iii) screening said at least one clone of the host cell for production of a mixture of antibodies having the desired effect by a functional assay; and
(iv) identifying at least one clone that produces a mixture of antibodies having the desired effect.

21. A method according to claim 20, wherein said host cell comprises a nucleic acid sequence encoding a common light chain that is capable of pairing with said at least two different heavy chains, such that the produced antibodies comprise common light chains.

22. A method according to claim 20 or 21, wherein said culturing in step ii) and said screening in step iii) is performed with at least two clones.

23. A method according to any one of claims 20-22, wherein said culturing of step ii) and/or said screening of step iii) is performed using high-throughput procedures.

24. A method according to any one of claims 20-23, wherein said host cell is capable of high-level expression

of proteins without the necessity of amplification of the nucleic acid encoding said proteins in said cell.

25. A method according to any one of claims 20-24, wherein said cell is derived from a human embryonic retinoblast that has been immortalized or transformed by adenoviral E1 sequences.

26. A method according to claim 25, wherein said host cell is derived from PER.C6.

27. A method according to any one of claims 20-26, wherein said mixture of antibodies comprises at least two antibodies having different specificities and/or affinities.

28. A method of producing a mixture of antibodies, comprising the step of:
(i) culturing a host cell clone identified by a method according to any one of claims 20-27 under conditions conducive to expression of the nucleic acids encoding the at least one light chain and the at least two different heavy chains.

29. A method according to claim 28, further comprising the step of:
(ii) recovering the antibodies from the host cell or the host cell culture.

30. A mixture of antibodies that is obtainable by a method according to claim 28 or 29.

31. A method for making a recombinant host cell for production of a mixture of antibodies, the method comprising the steps of:
introducing into said host cell a nucleic acid sequence encoding a light chain and nucleic acid sequences encoding at least three different heavy chains that are capable of pairing with said light chain, wherein said nucleic acid sequences are introduced consecutively or concomitantly.

32. A method for making a recombinant host cell for production of a mixture of antibodies, the method comprising the step of:
introducing nucleic acid sequences encoding at least two different heavy chains into a recombinant host cell comprising a nucleic acid sequence encoding a light chain capable of pairing with at least two of said heavy chains.

33. A transgenic non-human animal or a transgenic plant comprising a nucleic acid sequence encoding a light chain and a nucleic acid sequence or sequences encoding at least two different heavy chains that are capable of pairing with said light chain, wherein said nucleic acid sequences encoding said light and heavy chains are under the control of a tissue-specific promoter.

34. A mixture of antibodies obtainable from a transgenic animal or transgenic plant according to claim 33.

35. A pharmaceutical composition comprising a mixture of recombinantly produced antibodies and a suitable carrier, wherein at least two different heavy chains are

represented in said mixture of recombinantly produced antibodies.

36. A pharmaceutical composition according to claim 35, wherein said mixture comprises bispecific antibodies.

37. A mixture of antibodies wherein at least two different heavy chains are represented, for use in the treatment or diagnosis of a human or animal subject.

38. Use of a mixture of antibodies wherein at least two different heavy chains are represented, for the preparation of a medicament for use in the treatment or diagnosis of a disease or disorder in a human or animal subject.

39. A method for producing a mixture of antibodies comprising different isotypes from a host cell, the method comprising the step of:
expressing in a recombinant host cell a nucleic acid sequence encoding a light chain and nucleic acid sequences encoding at least two heavy chains of different isotype that are capable of pairing with said light chain.

40. A method according to claim 39, wherein said isotypes comprise at least an IgG and an IgA.

41. A method for indentifying a mixture of antibodies having a desired effect in a functional assay, comprising the steps of
i) adding a mixture of antibodies in a functional assay, and

ii) determining the effect of said mixture in said assay, wherein the antibodies in said mixture comprise a common light chain.

42. A method according to claim 41, wherein said mixture is comprised in a composition according to any one of claims 11-19.

43. A method according to any one of claims 1-7, 20-29, 39 or 40, or a recombinant host cell according to claim 10, or a method of making a recombinant host cell according to claim 31 or 32, or a transgenic non-human animal or transgenic plant according to claim 33, wherein the nucleic acid sequence or sequences encoding at least one of said light and/or heavy chains have been obtained by a method comprising at least one antibody display selection step.

44. A method for producing a mixture of antibodies that are capable of binding to a target, the method comprising the steps of: i) bringing an antibody display library comprising antibodies into contact with material comprising a target, ii) at least one step of selecting antibodies binding to said target, iii) identifying at least two antibodies binding to said target, wherein said at least two antibodies comprise a common light chain, iv) introducing a nucleic acid sequence encoding the light chain and a nucleic acid sequence or nucleic acid sequences encoding the heavy chains of said at least two antibodies into a host cell, v) culturing a clone of said host cell under conditions conducive to expression of said

nucleic acid sequences.

45. A method for producing a mixture of antibodies in a recombinant host, the method including the step of: expressing in a recombinant host cell a nucleic acid sequence encoding a common light chain and nucleic acid sequences encoding at least two different heavy chains that differ in the variable region and that are capable of pairing with said common light chain, and wherein said heavy chains further differ in their constant regions sufficiently to reduce or prevent pairing between the different heavy chains.

46. A method according to claim 45, wherein said heavy chains are of different isotype.

47. A method according to claim 46, wherein said different isotypes include at least IgG1 and IgG3.

48. A method according to claim 46, wherein said different isotypes include at least an IgG and an IgA isotype.

49. A mixture of antibodies obtainable by a method according to any one of claims 45-48.

50. A method for producing a mixture of antibodies comprising dimeric IgA isotype {$(IgA)_2$} antibodies in a recombinant host, wherein at least part of said dimeric IgA antibodies have different binding regions in each of the two IgA subunits, the method comprising the step of: expressing in a recombinant host cell a nucleic acid

sequence encoding a common light chain and nucleic acid sequences encoding at least two different heavy chains of IgA isotype capable of pairing to said common light chain.

51. A method for producing a mixture of antibodies comprising an IgM antibody having at least two different specificities, the method comprising the step of expressing in a recombinant host cell a nucleic acid sequence encoding a common light chain and nucleic acid sequences encoding at least two different heavy chains of IgM isotype, wherein said heavy chains are capable of pairing to said common light chain.

52. An IgA dimer, an IgM pentamer or an IgM hexamer having at least two different specificities.

Title: Recombinant production of mixtures of antibodies

Abstract

The invention provides methods for producing mixtures of antibodies from a single host cell clone. Thereto a nucleic acid sequence encoding a light chain, and nucleic acid sequences encoding different heavy chains are expressed in a recombinant host cell. The antibodies in the mixtures according to the invention suitably comprise identical light chains paired to different heavy chains capable of pairing to the light chain, thereby forming functional antigen binding domains. Mixtures of antibodies are also provided by the invention. Such mixtures can be used in a variety of fields.

Fig. 1

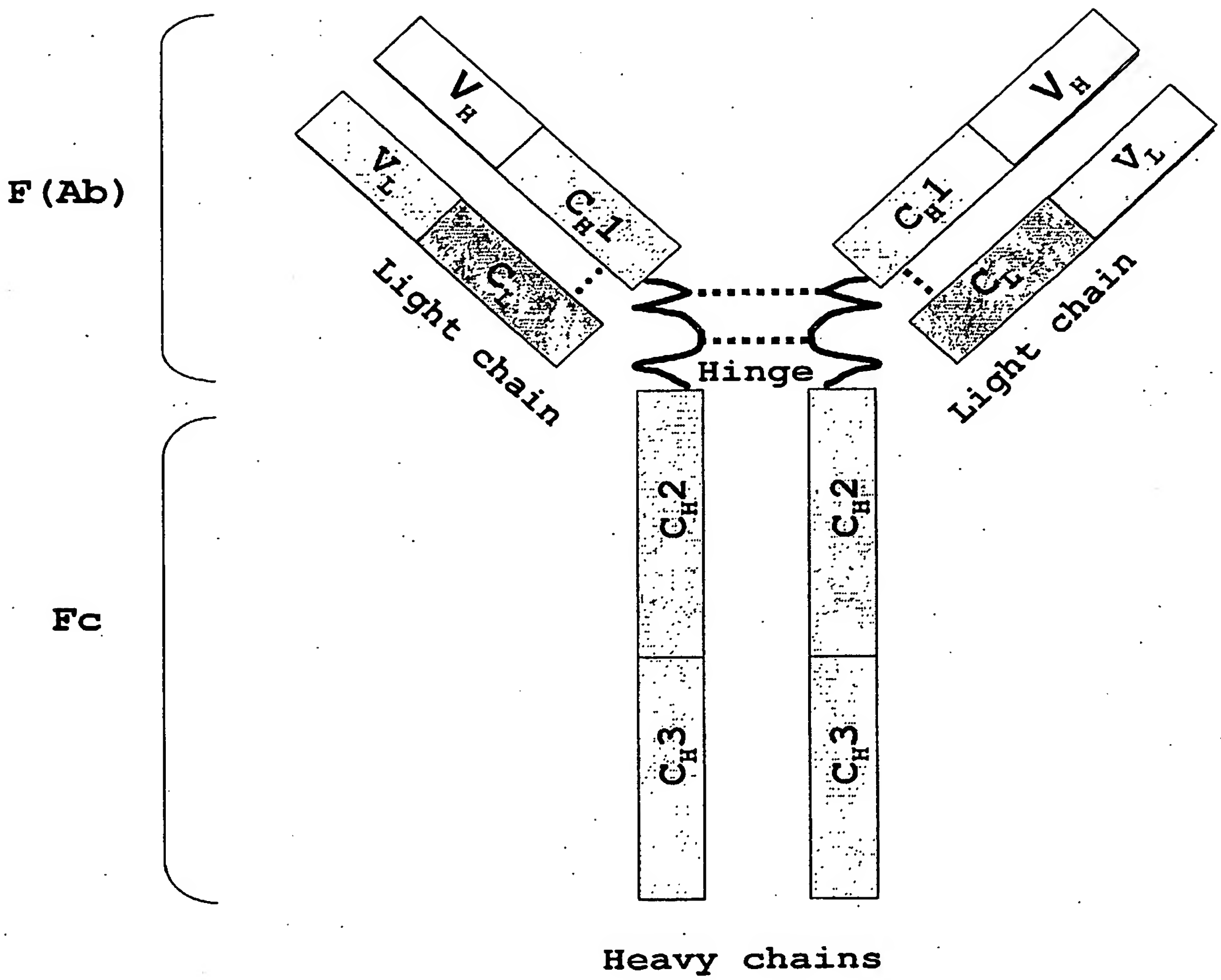


Fig. 2

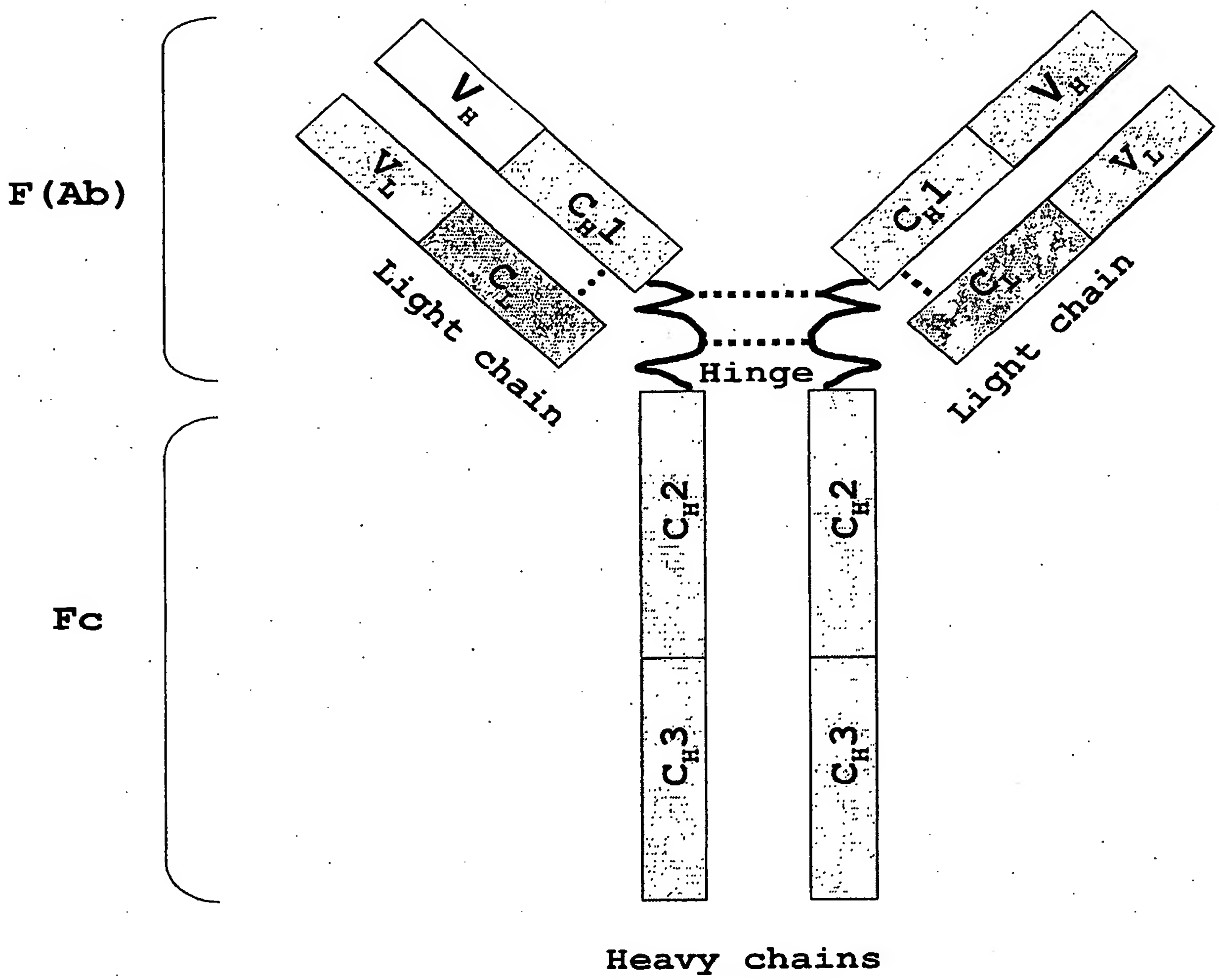


Fig. 3

```
UBS54-VL     GAAATTGAGCTCACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGCC
K53-VL       GAAATTGAGCTCACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGCC
             *********************************************************

UBS54-VL     TCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTGCATAGTAATGGATACAACTATTTGGAT
K53-VL       TCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTGCATAGTAATGGATACAACTATTTGGAT
             ************************************************************

UBS54-VL     TGGTACCTGCAGAAGCCAGGGCAGTCTCCACAGCTCCTGATCTATTTGGGTTCTAATCGG
K53-VL       TGGTACCTGCAGAAGCCAGGGCAGTCTCCACAGCTCCTGATCTATTTGGGTTCTAATCGG
             ************************************************************

UBS54-VL     GCCTCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAA
K53-VL       GCCTCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAA
             ************************************************************

UBS54-VL     ATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGCAAGCTCTACAAACT
K53-VL       ATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGCAAGCTCTACAAACT
             ************************************************************

UBS54-VL     TTCACTTTCGGCCCTGGGACCAAGGTGGAGATCAAA
K53-VL       TTCACTTTCGGCCCTGGGACCAAGGTGGAGATCAAA
             ************************************

UBS54-VH     CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAGG
K53-VH       CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAG
             ********************************************* **** **** *

UBS54-VH     GTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCGACAG
K53-VH       GTCTCCTGCAAGGCTTCTGGTTACACCTTTACCAGCTATGGTATCAGCTGGGTGCGACAG
             ********************   ****** * ******** *******************

UBS54-VH     GCCCCTGGACAAGGGCTTGAGTGGATGGGAGGGATCATCCCTATCTTTGGTACAGCAAAC
K53-VH       GCCCCTGGACAAGGGCTTGAGTGGATGGGATGGATCAGCGCTTACAATGGTAACACAAAC
             ****************************** ****** * **  *  *****   *****

UBS54-VH     TACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACGAATCCACGAGCACAGCC
K53-VH       TATGCACAGAAGCTCCAGGGCAGAGTCACCATGACCACAGACACATCCACGAGCACAGCC
             ** ********* **************** ** *** * ***  ****************

UBS54-VH     TACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCTGTGTATTACTGTGCAAGAGAC
K53-VH       TACATGGAGCTGAGGAGCCTGAGATCTGACGACACGGCCGTGTATTACTGTGCAAGGGGC
             ************** ************** ******** ***************** * *

UBS54-VH     CCGTTTC---------TTCACTATTGGGGCCAAGGTACCCTGGTCACCGTCTCGACA
K53-VH       ATGATGAGGGGTGTGTTTGACTACTGGGGCCAAGGTACCCTGGTCACCGTCTCGACA
               * *           ** **** *********************************
```

Fig. 4

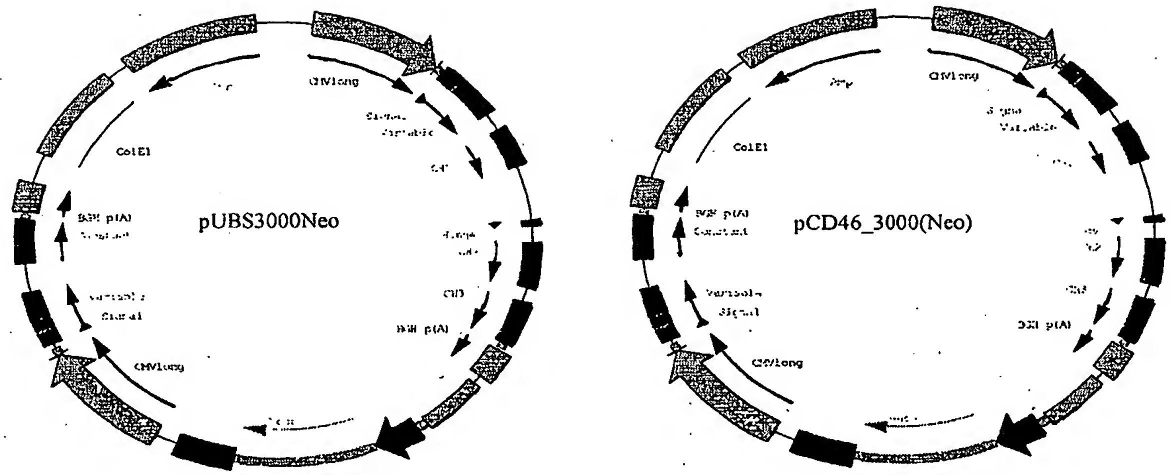
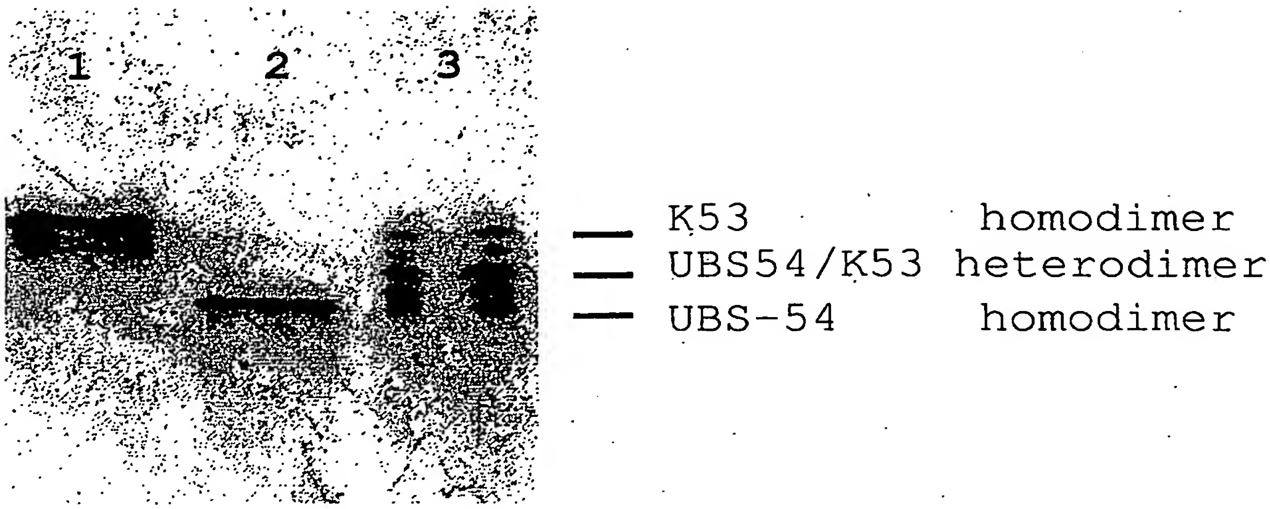

pUBS3000Neo
CMVlong
ColE1
BGH p(A)
CMVlong
pCD46_3000(Neo)
CMVlong
ColE1
BGH p(A)
Constant
Variable
Signal
CMVlong

Fig. 5

A.


1
2
3
K53 homodimer
UBS54/K53 heterodimer
UBS-54 homodimer

B.

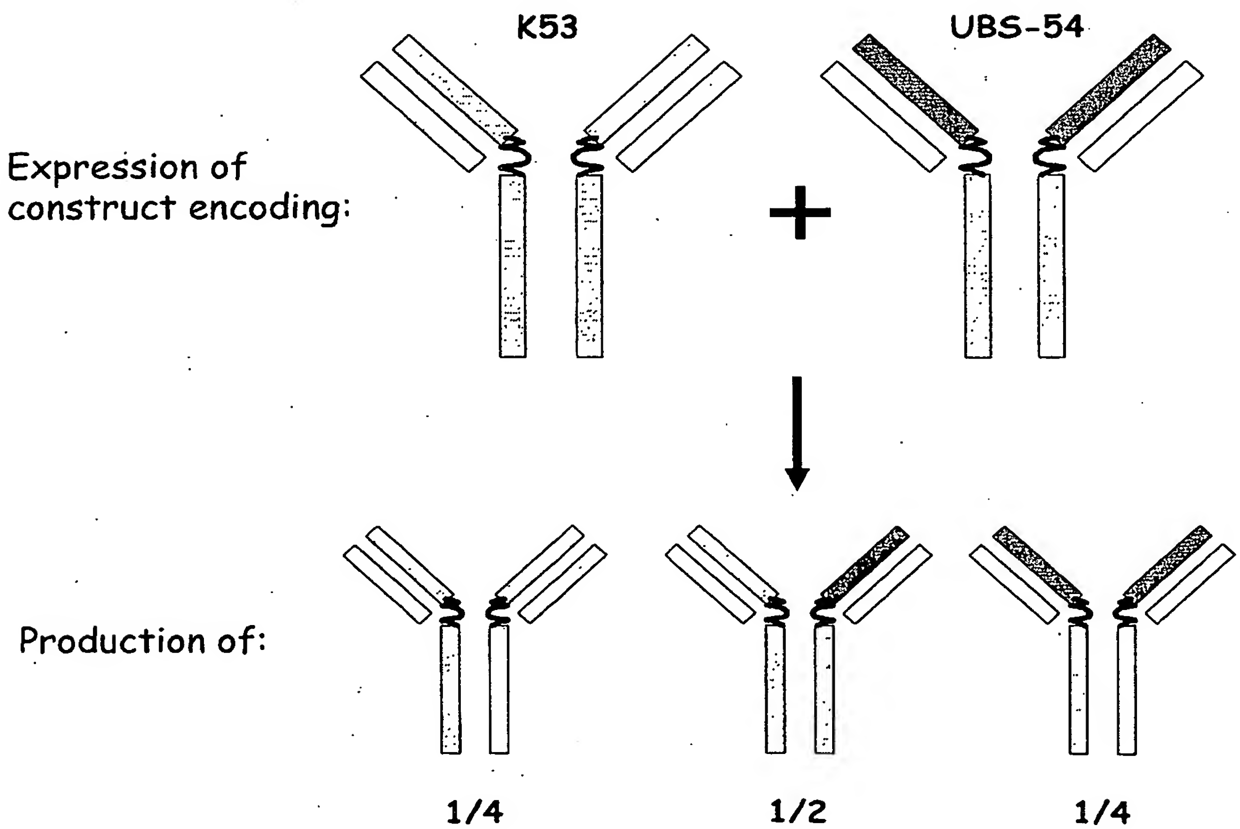

K53
UBS-54
Expression of construct encoding:
+
Production of:
1/4
1/2
1/4

Fig. 6

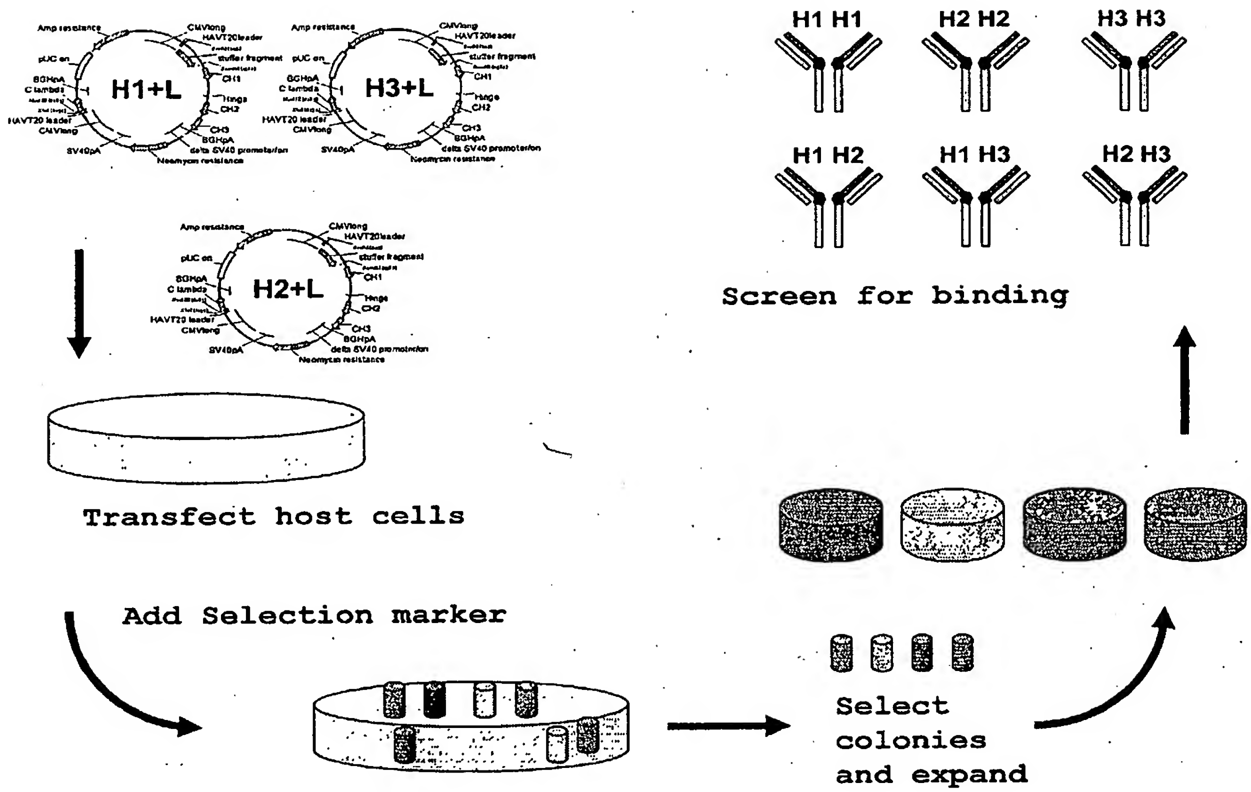

H1+L
H3+L
H2+L
H1 H1
H2 H2
H3 H3
H1 H2
H1 H3
H2 H3
Screen for binding
Transfect host cells
Add Selection marker
Select
colonies
and expand

Fig. 7

Anti-CD22 V_H fragment (Phage B28)

```
     M  A  E  V  Q  L  V  E  S  G  G  G  V  V  R  P  G  G  S  L  R  L  S  C·
1    ATGGCCGAGGTGCAGCTGGTGGAGTCTGGGGGAGGTGTGGTACGGCCTGGAGGGTCCCTGAGACTCTCCTG
     ·A  A  S  G  F  T  F  D  D  Y  G  M  S  W  V  R  Q  A  P  G  K  G  L  E·
72   TGCAGCCTCTGGATTCACCTTTGATGATTATGGCATGAGCTGGGTCCGCCAAGCTCCAGGGAAGGGGCTGG
     · W  V  S  G  I  N  W  N  G  G  S  T  G  Y  A  D  S  V  K  G  R  F  T
143  AGTGGGTCTCTGGTATTAATTGGAATGGTGGTAGCACAGGTTATGCAGACTCTGTGAAGGGCCGATTCACC
      I  S  R  D  N  A  K  N  S  L  Y  L  Q  M  N  S  L  R  A  E  D  T  A  V·
214  ATCTCCAGAGACAACGCCAAGAACTCCCTGTATCTGCAAATGAACAGTCTGAGAGCCGAGGACACGGCCGT
     ·Y  Y  C  A  R  G  F  L  R  F  A  S  S  W  F  D  Y  W  G  Q  G  T  L  V·
285  GTATTACTGTGCAAGAGGCTTTCTTCGTTTTGCTTCCTCCTGGTTTGACTATTGGGGCCAAGGTACCCTGG
     · T  V  S  R
356  TCACCGTCTCGAGA
```

Anti-CD72 V_H fragment (Phage II-2)

```
     M  A  Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  A  S  V  K  V  S  C··
1    ATGGCCCAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTTTCCTG
     ·K  A  S  G  Y  T  F  T  S  Y  Y  M  H  W  V  R  Q  A  P  G  Q  G  L  E·
72   CAAGGCATCTGGATACACCTTCACCAGCTACTATATGCACTGGGTGCGACAGGCCCCTGGACAAGGGCTTG
     · W  M  G  I  I  N  P  S  G  G  G  T  S  Y  A  Q  K  F  Q  G  R  V  T
143  AGTGGATGGGAATAATCAACCCTAGTGGTGGTGGCACAAGCTACGCACAGAAGTTCCAGGGCAGAGTCACC
     M  T  R  D  T  S  T  S  T  V  Y  M  E  L  S  S  L  R  S  E  D  T  A  V·
214  ATGACCAGGGACACGTCCACGAGCACAGTCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGT
     ·Y  Y  C  A  R  D  Y  Y  V  T  Y  D  S  W  F  D  S  W  G  Q  G  T  L  V·
285  GTATTACTGTGCAAGAGACTACTATGTTACGTATGATTCCTGGTTTGACTCCTGGGGCCAAGGTACCCTGG
     · T  V  S  R
356  TCACCGTCTCGAGA
```

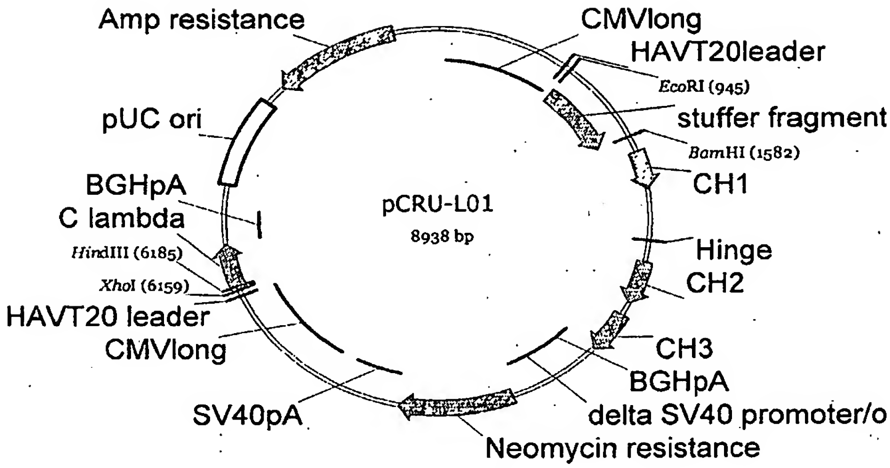
Anti-HLA-DR V_H fragment (Phage I-2)

```
     M  A  E  V  Q  L  V  E  S  G  G  G  L  V  Q  P  G  R  S  L  R  L  S  C·
1    ATGGCCGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGCAGGTCCCTGAGACTCTCCTG
     ·A  A  S  G  F  T  F  D  D  Y  A  M  H  W  V  R  Q  A  P  G  K  G  L  E·
72   TGCAGCCTCTGGATTCACCTTTGATGATTATGCCATGCACTGGGTCCGGCAAGCTCCAGGGAAGGGCCTGG
     · W  V  S  G  I  S  W  N  S  G  S  I  G  Y  A  D  S  V  K  G  R  F  T
143  AGTGGGTCTCAGGTATTAGTTGGAATAGTGGTAGCATAGGCTATGCGGACTCTGTGAAGGGCCGATTCACC
      I  S  R  D  N  A  K  N  S  L  Y  L  Q  M  N  S  L  R  A  E  D  T  A  V·
214  ATCTCCAGAGACAACGCCAAGAACTCCCTGTATCTGCAAATGAACAGTCTGAGAGCTGAGGACACGGCCGT
     ·Y  Y  C  A  R  D  L  Y  L  A  H  F  D  Y  W  G  Q  G  T  L  V  T  V  S·
285  GTATTACTGTGCAAGGGACCTTTATCTTGCGCATTTTGACTACTGGGGCCAAGGTACCCTGGTCACCGTCT
     · R
356  CGAGA
```

Shared V_L sequence of Phages I-2, II-2 and B28

```
     S  S  E  L  T  Q  D  P  A  V  S  V  A  L  G  Q  T  V  R  I  T  C  Q  G·
1    TCGTCTGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACATGCCAAGG
     ·D  S  L  R  S  Y  Y  A  S  W  Y  Q  Q  K  P  G  Q  A  P  V  L  V  I  Y·
72   AGACAGCCTCAGAAGCTATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCCCCTGTACTTGTCATCT
     · G  K  N  N  R  P  S  G  I  P  D  R  F  S  G  S  S  S  G  N  T  A  S
143  ATGGTAAAAACAACCGGCCCTCAGGGATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCTTCC
      L  T  I  T  G  A  Q  A  E  D  E  A  D  Y  Y  C  N  S  R  D  S  S  G  N·
214  TTGACCATCACTGGGGCTCAGGCGGAAGATGAGGCTGACTATTACTGTAACTCCCGGGACAGCAGTGGTAA
     ·H  V  V  F  G  G  G  T  K  L  T  V  L  G  A  A  A
285  CCATGTGGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGTGCGGCCGCA
```

Fig. 8.

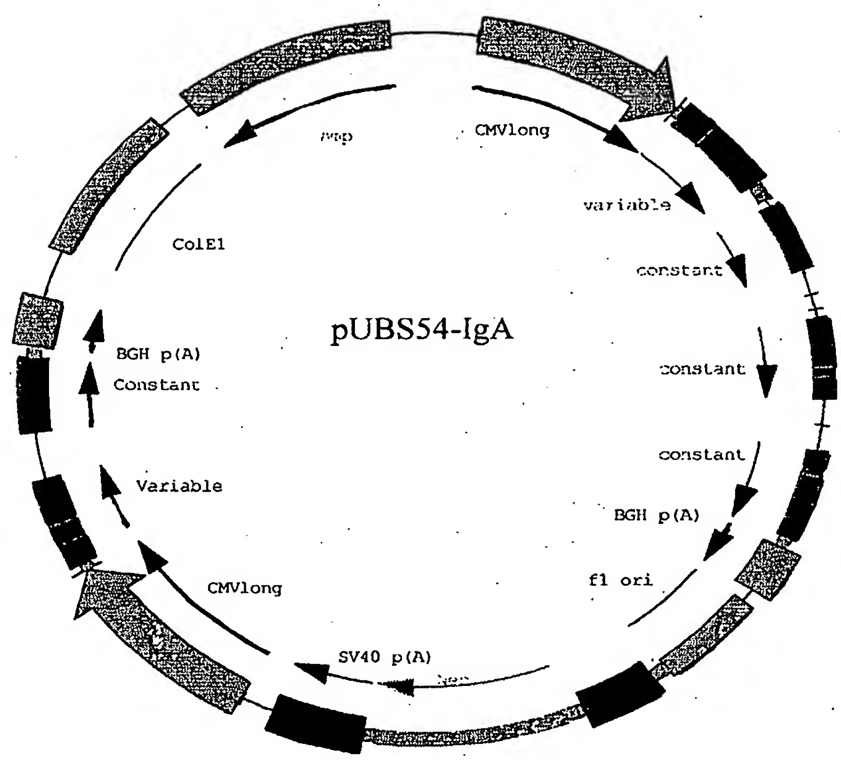


Vector pCRU-L01

```
   1  AATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCTAGGTGGTCAATATTGGCCATTA
  72  GCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGCTATTGGCCATTGCATACGTTGTATCCATA
 143  TCATAATATGTACATTTATATTGGCTCATGTCCAACATTACCGCCATGTTGACATTGATTATTGACTAGTT
 214  ATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACG
 285  GTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCAT
 356  AGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAG
 427  TACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCAT
 498  TATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC
 569  CATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTC
 640  TCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAAC
 711  AACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTT
 782  AGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGAT
 853  CCAGCCTCCGCGGCCGGGAACGGTGCATTGGAAGCTAGCCACCATGGCATGCCCTGGCTTCCTGTGGGCAC
 924  TTGTGATCTCCACCTGTCTTGAATTCTTATTAAGACTCCTTATTACGCAGTATGTTAGCAAACGTCGAAAA
 995  TACATACATAAAGGTGGCAACATATAAAAGAAACGCAAAGACACCACGGAATAAGTTTATTTTGTCACAAT
1066  CAATAGAAAATTCATATGGTTTACCAGCGCCAAAGACATAAGGGCGACATTCAACCGACTGAGGCAAAGAA
1137  GGTAAATATTGACGGAAATTATTCATTAAAGGTGAATTATCACCGTCACCGACTTGAGCCATTTGGGAATT
1208  AGAGCCAGCAAAATCACCAGTAGCACCATTACCATTAGCAAGGCCGGAAACGTCACCAATGAAACCATCGA
1279  TAGCAGCACCGTAATCAGTAGCGACAGAATCAAGTTTGCCTTTAGCGTCAGACTGTAGCGCGTTTTCATCG
1350  GCATTTTCGGTCATAGCCCCCTTATTAGCGTTTGCCATTTTTTCATAATCAAAATCACCGGAACCGGAGCC
1421  GCCACCGGAACCGCCACCCTCAGAGCCGCCACCCTCAGAACCGCCACCCTCAGAGCCGCCACCCTCAGAGC
1492  CGCCACCAGAACCACCACCAGAGCCGCCGCCAGCATTGACAGGAGGTTGAGGCAGGTCAGACGATTGGCCT
1563  TGATATTCACAAACGAATGGATCCGCGAGCCCAGACACTGGACGCTGAACCTCGCGGACAGTTAAGAACCC
1634  AGGGGCCTCTGCGCCCTGGGCCCAGCTCTGTCCCACACCGCGGTCACATGGCACCACCTCTCTTGCAGCCT
1705  CCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTG
1776  GGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGG
1847  CGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCT
1918  CCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAG
1989  AGAGTTGGTGAGAGGCCAGCACAGGGAGGGAGGGTGTCTGCTGGAAGCCAGGCTCAGCGCTCCTGCCTGGA
2060  CGCATCCCGGCTATGCAGTCCCAGTCCAGGGCAGCAAGGCAGGCCCCGTCTGCCTCTTCACCCGGAGGCCT
2131  CTGCCCGCCCCACTCATGCTCAGGGAGAGGGTCTTCTGGCTTTTTCCCCAGGCTCTGGGCAGGCACAGGCT
2202  AGGTGCCCCTAACCCAGGCCCTGCACACAAAGGGGCAGGTGCTGGGCTCAGACCTGCCAAGAGCCATATCC
2273  GGGAGGACCCTGCCCCTGACCTAAGCCCACCCCAAAGGCCAAACTCTCCACTCCCTCAGCTCGGACACCTT
2344  CTCTCCTCCCAGATTCCAGTAACTCCCAATCTTCTCTCTGCAGAGCCCAAATCTTGTGACAAAACTCACAC
2415  ATGCCCACCGTGCCCAGGTAAGCCAGCCCAGGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAG
2486  TAGCCTGCATCCAGGGACAGGCCCCAGCCGGGTGCTGACACGTCCACCTCCATCTCTTCCTCAGCACCTGA
2557  ACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCC
2628  CTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC
2699  GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAG
2770  CGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
2841  TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGTGGGACCCGTGGGGTGCGAGGGCCACATGGA
2912  CAGAGGCCGGCTCGGCCCACCCTCTGCCCTGAGAGTGACCGCTGTACCAACCTCTGTCCCTACAGGGCAGC
2983  CCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACC
3054  TGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAA
3125  CTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACA
3196  AGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACG
3267  CAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGAGTTTCTAGAGCCTCGACTGTGCCTTCTAGTTGCCAGCC
3338  ATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAAT
3409  AAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGAC
3480  AGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGATGCGGTGGGCTCTATGGCTTCTGAGGC
```

3551 GGATCTGGGTGTGGAAAGTCCCCAGGCTCCCCAGGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGT
3622 CAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAG
3693 TCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCC
3764 GCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCTGCCTCTGAGCTATTCCAGA
3835 AGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTCCCGGGAGCTTGGATATCCATTTTC
3906 GGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCC
3977 GGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCG
4048 TGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAA
4119 CTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGT
4190 TGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACC
4261 TTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACC
4332 TGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGA
4403 TCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCA
4474 TGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGC
4545 CGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTAC
4616 CCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTC
4687 CCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGGTG
4758 CTACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGG
4829 CTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCAACTTGTTTATTGCAGCTT
4900 ATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGT
4971 TGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGTATACCGTCGACCTCTAGCTAGAGCTTGGC
5042 GTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCG
5113 GAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG
5184 CCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGC
5255 GCTGCTTCGCTAGGTGGTCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATAT
5326 TGGCTATTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGTCCAACAT
5397 TACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGC
5468 CCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCG
5539 CCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGG
5610 TGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATT
5681 GACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTG
5752 GCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTG
5823 GATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCAC
5894 CAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGT
5965 ACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCT
6036 GTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAATCGAT
6107 GCCACCATGGCATGCCCTGGCTTCCTGTGGGCACTTGTGATCTCCACCTGTCTCGAGTTTTCCGGATTTGC
6178 GGCCGCAAGCTTACCGTGCTGGGCCAGCCCAAGGCCGCTCCCAGCGTGACCCTGTTCCCCCCCTCCTCCGA
6249 GGAGCTGCAGGCCAACAAGGCCACCCTGGTGTGCCTCATCAGCGACTTCTACCCTGGCGCCGTGACCGTGG
6320 CCTGGAAGGCCGACAGCAGCCCCGTGAAGGCCGGCGTGGAGACCACCACCCCCAGCAAGCAGAGCAACAAC
6391 AAGTACGCCGCCAGCAGCTACCTGAGCCTCACCCCCGAGCAGTGGAAGAGCCACCGGAGCTACAGCTGCCA
6462 GGTGACCCACGAGGGCAGCACCGTGGAGAAGACCGTGGCCCCCACCGAGTGCAGCTAGACTTAAGTCGAGT
6533 CAGCCTCGACTGTTCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTG
6604 GAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCA
6675 TTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTG
6746 GGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGCATTAATGAATCGGCCAACGCGCGG
6817 GGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGG
6888 CTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGG
6959 AAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC
7030 ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGA
7101 CTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTAC
7172 CGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCA
7243 GTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCC
7314 TTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGG
7385 TAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCT
7456 ACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGC
7527 TCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAA
7598 AAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTT
7669 AAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTT
7740 AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTAT
7811 CTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGG
7882 AGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCA
7953 GCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTC
8024 TATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTG
8095 CTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGG
8166 CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAG
8237 TAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCG
8308 TAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGT
8379 TGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGG
8450 AAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTC
8521 GTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAA
8592 AATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTA
8663 TTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAA
8734 TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCGACGGATCGGGAGATCTCCCGATCCCC
8805 TATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTG
8876 TTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGAC

Fig. 9



pUBS54-IgA
CMVlong
variable
constant
constant
constant
BGH p(A)
f1 ori
SV40 p(A)
CMVlong
Variable
Constant
BGH p(A)
ColE1

Fig. 10

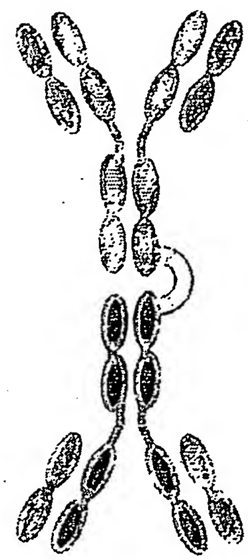


Fig. 11

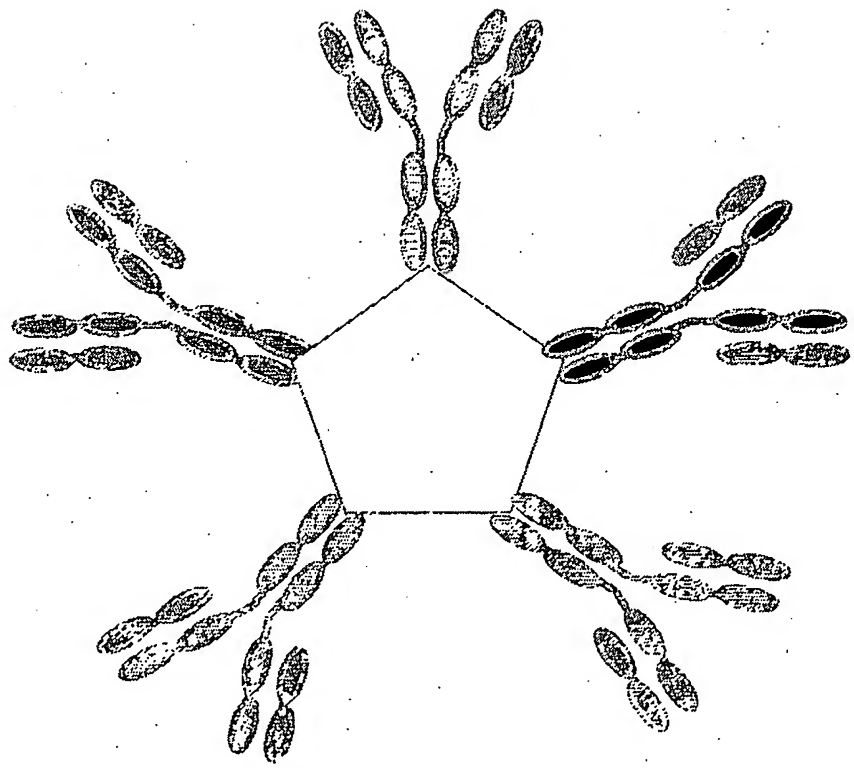


Fig. 12

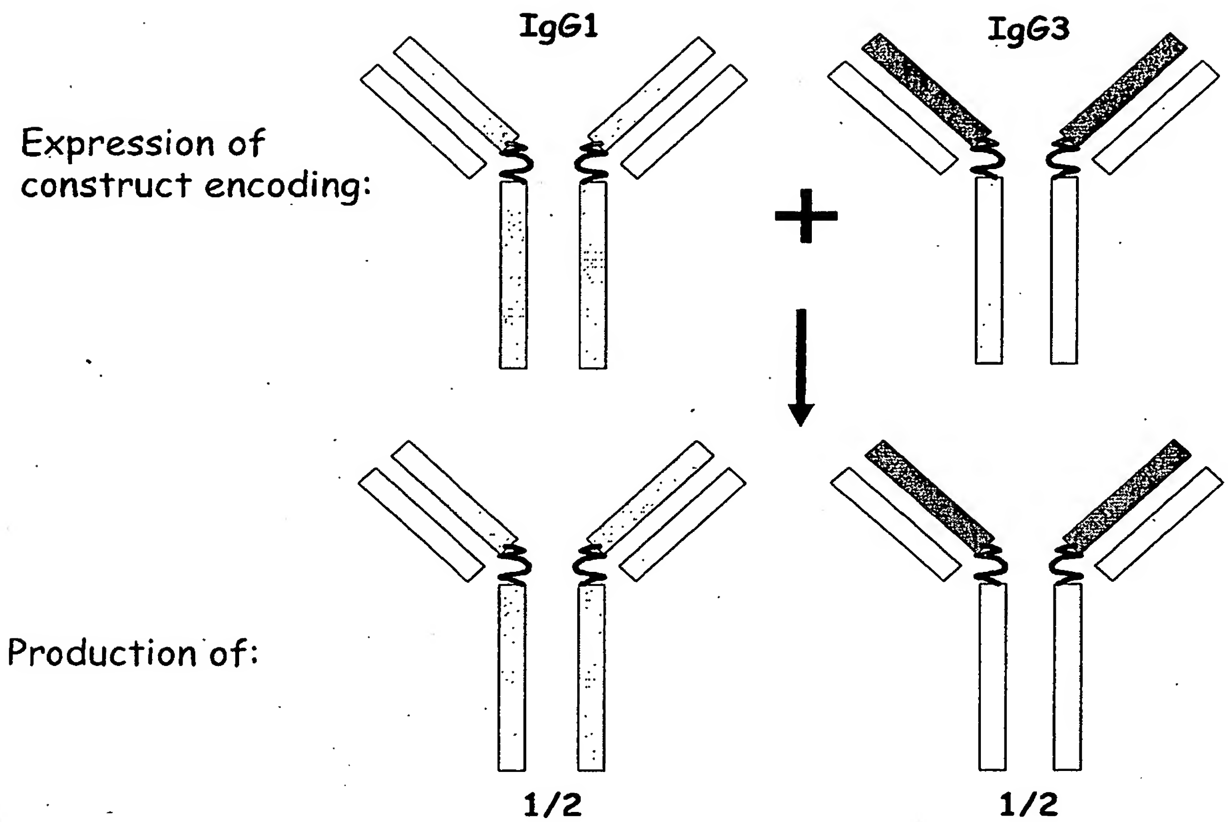
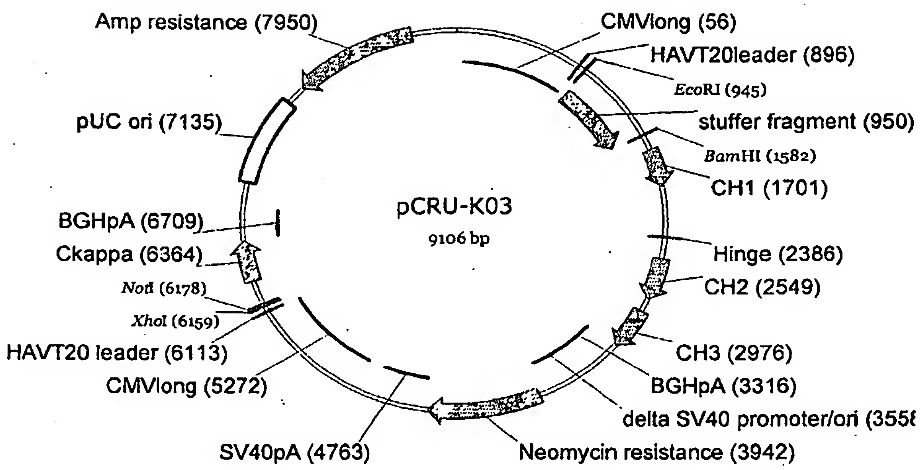

IgG1
IgG3
Expression of construct encoding:
+
Production of:
1/2
1/2

Fig. 13



1 VECTOR PCRU-K03

```
   1   AATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCTAGGTGGTCAATATTGGCCATTA
  72   GCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGCTATTGGCCATTGCATACGTTGTATCCATA
 143   TCATAATATGTACATTTATATTGGCTCATGTCCAACATTACCGCCATGTTGACATTGATTATTGACTAGTT
 214   ATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACG
 285   GTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCAT
 356   AGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAG
 427   TACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCAT
 498   TATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC
 569   CATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTC
 640   TCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAAC
 711   AACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTT
 782   AGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGAT
 853   CCAGCCTCCGCGGCCGGGAACGGTGCATTGGAAGCTAGCCACCATGGCATGCCCTGGCTTCCTGTGGGCAC
                                    EcoRI
                                    ~~~~~~
 924   TTGTGATCTCCACCTGTCTTGAATTCTTATTAAGACTCCTTATTACGCAGTATGTTAGCAAACGTCGAAAA
 995   TACATACATAAAGGTGGCAACATATAAAAGAAACGCAAAGACACCACGGAATAAGTTTATTTTGTCACAAT
1066   CAATAGAAAATTCATATGGTTTACCAGCGCCAAAGACATAAGGGCGACATTCAACCGACTGAGGCAAAGAA
1137   GGTAAATATTGACGGAAATTATTCATTAAAGGTGAATTATCACCGTCACCGACTTGAGCCATTTGGGAATT
1208   AGAGCCAGCAAAATCACCAGTAGCACCATTACCATTAGCAAGGCCGGAAACGTCACCAATGAAACCATCGA
1279   TAGCAGCACCGTAATCAGTAGCGACAGAATCAAGTTTGCCTTTAGCGTCAGACTGTAGCGCGTTTTCATCG
1350   GCATTTTCGGTCATAGCCCCCTTATTAGCGTTTGCCATTTTTTCATAATCAAAATCACCGGAACCGGAGCC
1421   GCCACCGGAACCGCCACCCTCAGAGCCGCCACCCTCAGAACCGCCACCCTCAGAGCCGCCACCCTCAGAGC
1492   CGCCACCAGAACCACCACCAGAGCCGCCGCCAGCATTGACAGGAGGTTGAGGCAGGTCAGACGATTGGCCT
                                 BamHI
                                 ~~~~~~
1563   TGATATTCACAAACGAATGGATCCGCGAGCCCAGACACTGGACGCTGAACCTCGCGGACAGTTAAGAACCC
1634   AGGGGCCTCTGCGCCCTGGGCCCAGCTCTGTCCCACACCGCGGTCACATGGCACCACCTCTCTTGCAGCCT
1705   CCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTG
1776   GGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGG
1847   CGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCT
1918   CCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAG
1989   AGAGTTGGTGAGAGGCCAGCACAGGGAGGGAGGGTGTCTGCTGGAAGCCAGGCTCAGCGCTCCTGCCTGGA
2060   CGCATCCCGGCTATGCAGTCCCAGTCCAGGGCAGCAAGGCAGGCCCCGTCTGCCTCTTCACCCGGAGGCCT
2131   CTGCCCGCCCCACTCATGCTCAGGGAGAGGGTCTTCTGGCTTTTTCCCCAGGCTCTGGGCAGGCACAGGCT
2202   AGGTGCCCCTAACCCAGGCCCTGCACACAAAGGGGCAGGTGCTGGGCTCAGACCTGCCAAGAGCCATATCC
2273   GGGAGGACCCTGCCCCTGACCTAAGCCCACCCCAAAGGCCAAACTCTCCACTCCCTCAGCTCGGACACCTT
2344   CTCTCCTCCCAGATTCCAGTAACTCCCAATCTTCTCTCTGCAGAGCCCAAATCTTGTGACAAAACTCACAC
2415   ATGCCCACCGTGCCCAGGTAAGCCAGCCCAGGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAG
2486   TAGCCTGCATCCAGGGACAGGCCCCAGCCGGGTGCTGACACGTCCACCTCCATCTCTTCCTCAGCACCTGA
2557   ACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCC
2628   CTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC
2699   GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAG
2770   CGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC
2841   TCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGTGGGACCCGTGGGGTGCGAGGGCCACATGGA
2912   CAGAGGCCGGCTCGGCCCACCCTCTGCCCTGAGAGTGACCGCTGTACCAACCTCTGTCCCTACAGGGCAGC
2983   CCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACC
3054   TGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAA
3125   CTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACA
3196   AGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACG
3267   CAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGAGTTTCTAGAGCCTCGACTGTGCCTTCTAGTTGCCAGCC
```

3338 ATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAAT
3409 AAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGAC
3480 AGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGC
3551 GGATCTGGGTGTGGAAAGTCCCCAGGCTCCCCAGGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGT
3622 CAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAG
3693 TCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCC
3764 GCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCTGCCTCTGAGCTATTCCAGA
3835 AGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTCCCGGGAGCTTGGATATCCATTTTC
3906 GGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCC
3977 GGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCG
4048 TGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAA
4119 CTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGT
4190 TGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACC
4261 TTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACC
4332 TGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGA
4403 TCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCA
4474 TGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGC
4545 CGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTAC
4616 CCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTC
4687 CCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGGTG
4758 CTACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGG
4829 CTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCAACTTGTTTATTGCAGCTT
4900 ATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGT
4971 TGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGTATACCGTCGACCTCTAGCTAGAGCTTGGC
5042 GTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCG
5113 GAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG
5184 CCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGC
5255 GCTGCTTCGCTAGGTGGTCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATAT
5326 TGGCTATTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGTCCAACAT
5397 TACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGC
5468 CCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCG
5539 CCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGG
5610 TGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATT
5681 GACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTG
5752 GCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTG
5823 GATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCAC
5894 CAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGT
5965 ACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCT
6036 GTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAATCGAT

XhoI NotI
~~~~~~ ~~

6107 GCCACCATGGCATGCCCTGGCTTCCTGTGGGCACTTGTGATCTCCACCTGTCTCGAGTTTTCCGGATTTGC

NotI
~~~~~~

6178 GGCCGCTAGGAAGAAACTCAAAACATCAAGATTTTAAATACGCTTCTTGGTCTCCTTGCTATAATTATCTG
6249 GGATAAGCATGCTGTTTTCTGTCTGTCCCTAACATGCCCTGTGATTATCCGCAAACAACACACCCAAGGGC
6320 AGAACTTTGTTACTTAAACACCATCCTGTTTGCTTCTTTCCTCAGGAACTGTGGCTGCACCATCTGTCTTC
6391 ATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTA
6462 TCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCA
6533 CAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAG
6604 AAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAG
6675 GGGAGAGTGTTAGACTTAAGTCGAGTCAGCCTCGACTGTTCCTTCTAGTTGCCAGCCATCTGTTGTTTGCC
6746 CCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATT
6817 GCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGA
6888 TTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCT
6959 GCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCA
7030 CTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTA
7101 TCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAA
7172 AAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAA
7243 GTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGC
7314 TCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTC
7385 TCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAAC
7456 CCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGAC
7527 TTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTT
7598 CTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAG
7669 TTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTTTTTTTGTT
7740 TGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGA
7811 CGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGA
7882 TCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTAC
7953 CAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCC
8024 CGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACC
8095 CACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCT
8166 GCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAA
8237 TAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCAT
8308 TCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCC
8379 TTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCA
8450 TAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCT

8521 GAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC
8592 AGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTT
8663 GAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT
8734 CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATA
8805 CTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATT
8876 TGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCG
8947 ACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAA
9018 GCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACA
9089 AGGCAAGGCTTGACCGAC